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Proceedings

The 4th International Carnivorous Plant Conference

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June 21 ~ June 23, 2002

Proceedings of the 4th International Carnivorous Plant Conference, Tokyo

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Cover: *Dionaea muscipula* cv. 'Kinchyaku' (=means purse), which has imperfect trap-closure but somewhat quite similar to *Drosera* trap movement, was produced by Katsuhiko Kondo using a gene engineering *in vitro* condition in 1999 and is now under mass-propagation.

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Edited by Katsuhiko Kondo

Organized by the International Carnivorous Plant Society and
Organizing Committee of the 4th International Carnivorous Plant Conference

Greetings

"Welcome CP enthusiasts to the 4th International Carnivorous Plant Conference, Tokyo." This Conference of the International Carnivorous Plant Society is incorporated with National Science Museum and Insectivorous Plant Society, Japan, which was founded in November 20, 1949 and thus, is the oldest among the world CP societies. This Conference is the first of the series for the 21st Century, the first in the Asian region, and 30 years anniversary of our CPN (Volume 31 in 2002). We are very grateful in this event to have many participants from all over the world. We feel this Conference is just like an alumni and alumnae association because most of us have already known each other through CPs, CP research papers and other foundations. We have in this Conference wide diversity of invited oral presentations, public poster resentations, workshops, CP exhibitions and Cps and CP books for sale. We try our best to arrange this, truly memorable event. We must express our heartfelt appreciation for your generous support and warm collaboration during the Conference. The program should influence CP enthusiasm to popularity, get more interests in CPs, and grow international exchanges of ideas, informations, research properties, and live plants as well. Let me offer my wishes for a fruitful outcome to this grand event and hope all of you happy and rewarding stay in Tokyo and excursion to Mt. Koushin.

Katsuhiko Kondo
Chairperson,
Organizing Committee
The 4th International
Carnivorous Plant Conference

The 4th International Carnivorous Plant Conference

Tokyo, Japan 2002

Mother Organization: The Fourth International Carnivorous Plant Society Conference

In cooperation with: **National Science Museum**; and **Insectivorous Plant Society**, Japan
Under the auspices of: **Japanese Ministry of Education, Science, Sports, Culture and Technology**; and **Botanical Gardens Association of Japan**
Sponsored by: **Hiroshima University Supporter's Association and Satake Foundation**

Editor: **Katsuhiko Kondo**

THE FOURTH INTERNATIONAL CARNIVOROUS PLANT CONFERENCE

PROGRAM

June 21 (Friday)

8:00 **Registration and Check In**

8:30 **Opening Address**
Kondo, Katsuhiko (4th International Carnivorous Plant Conference; Hiroshima University, Japan)
Konishi, Tatsuo (National Science Museum, Japan)
Komiya, Sadashi (Insectivorous Plant Society, Japan)

9:00 Clark, Charles (Hong Kong University of Science and Technology, Hong Kong)
"Twelve years of ecological research on *Nepenthes* in Southeast Asia-Some personal highlight"

9:30 **Keynote Lecture**
Hasebe, Mitsuyasu (National Institute for Basic Biology; and The Graduate University for Advanced Studies, Japan)
"Phylogeny of sundews, *Drosera* (Droseraceae) based on chloroplast *rbcL* and nuclear 18S ribosomal DNA sequences"

10:30～Coffee Break

11:00 Adam, Jummat (University of Kebangsaan Malaysia, Malaysia)
"Population structure of *Nepenthes* species (pitcher plants) from Weston, Sipitang in Sabah"

11:40 **Keynote Lecture**
Hotta, Mitsuru (Kagoshima University; Kagoshima Prefectural College, Japan) (VIDEO)
"*Nepenthes* in Malaysia"

12:00～Lunch

13:30 Lee, Chi'en (Malaysiana Tropicals, Malaysia)
"*Nepenthes* species of the Hose Mountains in Sarawak, Borneo"

14:00 Hoshi, Yoshikazu (Ariake National College of Technology, Japan)
"Chromosome studies in *Drosera* (Droseraceae)"

14:30 Darnowski, Douglas W. (Washington College, U.S.A.)
"Observations on the associations of crustaceans and protists with actively growing *Aldrovanda vesiculosa*"

15:30 Hartmeyer, Siggi (Germany) (VIDEO)
"Observations on three Australian *Drosera* species of Sect. Arachnopus, Lasiocephala and Prolifera (Droseraceae), using digital video as medium"

16:30 Shibata, Chiaki (Nippon Dental University, Japan) (VIDEO)
"Four seasons of *Pinguicula ramosa* and its habitat of Mt.Koushin"

17:00 Drink Break

19:00～ **Workshop 1** at the Conference Hall, National Olympic Memorial Youth Center
"Commercial utilization of carnivorous plants for full seasons" (tentative title)

June 22 (Saturday)

8:30 Joel, Daniel M. (Newe-Ya'ar Research Center, Israel; Utsunomiya University)

"Carnivory and parasitism in plants"

9:00 Schlauer, Jan (Tuebingen University, Germany)
"Global carnivorous plant diversity-Insights and incentives"

9:30～Coffee Break

10:00 Legendre, L. L. (University of Western Sydney, Australia)
"Evolution of carnivory in the Lentibulariaceae"

10:30 Owen, T. Page, Jr. (Connecticut College, U.S.A.)
"The structural basis for nutrient transport in the pitchers of *Nepenthes*"

11:00 **Poster Session**

12:30～Lunch

14:00～17:3 **Symposium Chairperson: Joel, Daniel M.**
"Leaf-blade movement of Droseraceae members"

(1) Keynote Lecture
Williams, Stephen E. (Lebanon Valley College, U.S.A.)
"Comparative physiology of the Droseraceae *sensu stricto*. How do tentacles bend and traps close?"

(2) Keynote Lecture
Fagerberg, Wayne R. (University of New Hampshire, U.S.A.)
"Changes in trap tissue relationships during closure·reopening in venus's flytrap (*Dionaea muscipula* Ellis): A possible model to explain trap morphological changes"

(3)
Kondo, Katsuhiko (Hiroshima University, Japan)
"Open-closure trap system modeled in tissue-cultured *Dionaea muscipula* controled by certain chemical substance in culture media"

(4)
Ueda, Minoru (Keio University, Japan)
"Chemical substance concerning the leaf movement of *Dionaea muscipula*"

17:45～Commemorative photographing

18:00～Banquet

20:00 **Workshop 2** at the Conference Hall, the National Olympic Memorial Youth Center
"Conservation, protection laws, and so on for native carnivorous plants" (tentative title)

June 23 (Sunday)

8:30 Song, Leo (California State University, Fullerton, U.S.A.)
"The CP Collection at California State University, Fullerton: An exercise in relevance"

9:00 Wistuba, Andreas (Germany)
"Recent observations on the genus *Heliamphora* (Sarraceniaceae)"

10:00 Kurata, Shigeo (Japan)
"Revision trial in recent enumeration of *Nepenthes* species"

10:30 Takahashi, Kenji (Japan)
"Purification and enzymatic characterization of an aspartic proteinase (Nepenthesin) from the insectivorous plant Nepenthes distillatoria"

11:00 Murugan, C. (Madurai Kamaraj University, Madurai, India)

(possible)

11:30 **Clothing Address**
Katsuhiko Kondo, Tatsuo Konishi, and Sadashi Komiya
Get-together Farewell Snack

After
12:00 **Excursion to Mt. Koushin** till evening on June 24
(Responsible persons: Sadashi Komiya, Chiaki Shibata and Hiro Shimai)

June 25 **Post-Conference Optional tours to**: (1) *Aldrovanda* pond and a personal site for artificial tetraploidized *Byblis* (Responsible persons: Sadashi Komiya and Chiaki Shibata). (2) Nansou Carnivorous Plant Garden (Responsible person: Yukio Koshikawa)

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Twelve years of Ecological Research on *Nepenthes* in Southeast Asia - Some personal highlights.

Charles Clarke

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Abstract. I first started performing ecological research on *Nepenthes* in 1989 in Brunei, Borneo. Since then, ecological, biogeographical and taxonomic research on *Nepenthes* have dominated my academic life. The recent publication of my second text, *Nepenthes of Sumatra & Peninsular Malaysia*, marks the end of a major phase of my research career, and this presentation aims to summarise some of the more enjoyable and interesting work I have done. My primary interest in the ecology of *Nepenthes* has always been the structure, assembly and dynamics of the communities of animals which live inside *Nepenthes* pitchers. This work has shown that animal community structure is related to a wide variety of factors, ranging from host-pitcher characteristics to broad-scale geographical processes. The unique interaction between the swimming ant, *Camponotus schmitzi* and its host, *Nepenthes bicalcarata* was described in detail for the first time. Explorations of highland habitats in Borneo yielded much anecdotal information for possible investigation in future years. Recently, my research has focussed on the *Nepenthes* from Sumatra & Peninsular Malaysia. While I was researching this text, no fewer than five new *Nepenthes* taxa were discovered or described from these regions. Further interesting ecological observations were made, in particular, the unusual trapping mechanism of *Nepenthes inermis*, which seems to be part pitfall, part flypaper. Although many of these observations await formal investigation, it is clear that our knowledge of *Nepenthes* in Sumatra and Borneo is much less extensive than we thought, and it is now time to attempt more rigorous field-based experiments in order to better understand the ecology and taxonomy of these wonderful plants.

Introduction

Nepenthes are the famous tropical pitcher plants, producing jug-shaped "pitchers" that are designed to attract, trap and digest small animals. They are common in habitats which are deficient in nutrients (particularly nitrogen), such as swamps, bogs and mountain summits. They make up for the shortfall in soil nutrients by trapping and digesting animals. The nutrients obtained by this process are transported to other parts of the plant to help them to grow and reproduce. This provides them with a competitive advantage over "normal" plants.

Most *Nepenthes* species are found in South-east Asia, particularly in the Sunda region, which includes Borneo, Sumatra, the Malay Peninsula, Java and some of the southern islands of the Philippines. Outlying species occur in Madagascar, the Seychelles, India, Sri Lanka, New Caledonia, Australia and southern China. Secondary centres of diversity include New Guinea, the Philippines and Sulawesi. *Nepenthes mirabilis* has by far the widest geographical distribution of any species, extending from southern China to northern Australia. In contrast, most species have very restricted distributions, and several have only been recorded from single localities.

For convenience, scientists usually divide *Nepenthes* into two major groups, based on the altitudes at which they grow. *Lowland* species are generally confined to altitudes below 1000 m above sea level (a.s.l.), whereas *highland* species are usually found above 1000 m a.s.l. The lowland species are usually found in three major habitat types: tropical heath forest, peat swamp forest and secondary vegetation. *Nepenthes* may be very abundant in secondary habitats which remain moist throughout the year and have poor, acidic soils.

Tropical lowland evergreen rain forests usually occur from sea level up to 1000 or 1500 m a.s.l. Above these altitudes they are replaced by montane forests. The most

remarkable montane habitats are the mossy forests, where the trunks and branches of trees are gnarled and festooned with mosses. Highland *Nepenthes* species are particularly abundant in these habitats, especially in sunny areas, such as ridge crests and mountain summits. Many of the highland species are epiphytes, and sometimes the only clue to their presence is a few dead pitchers which have fallen to the ground from the forest canopy.

As well as trapping and digesting insects for nutritional benefit, it has been shown that the pitchers of most *Nepenthes* species also provide habitats for a range of aquatic invertebrates (Beaver 1983, 1985, Clarke and Kitching 1993). The majority of the macrofauna are dipterans, forming discreet communities which have proved useful in food web research. Beaver (1979) classified the organisms found in *Nepenthes* pitchers into three groups. Nepenthebiont species are those which live or develop only within the fluid of *Nepenthes* pitchers: they depend on pitchers for their survival. Nepenthephiles are those species which live or develop primarily in *Nepenthes* pitchers, but are occasionally found in other habitats as well. Nepenthexenes are not usually found in *Nepenthes* pitchers, but occasionally colonise them, for a variety of reasons (see Beaver, 1983).

Some of the organisms that have been found living in *Nepenthes* pitchers include spiders, tadpoles, mites, and larvae of mosquitoes, midges and flies. Most pitchers are colonised 4-5 species of different organisms, but in some cases, the communities can be remarkably diverse. For example, in *Nepenthes bicalcarata* pitchers in Brunei, I found a total of 33 species of infaunal organisms (Clarke, 1998a; Clarke and Kitching, 1993).

These communities are of interest to ecologists who study food web dynamics, as these simple communities are highly replicated in space and time, and the host plants are found in a variety of different habitats. This makes it comparatively easy to design and perform experiments on animals communities that would be difficult, if not impossible, to perform elsewhere.

The taxonomy of *Nepenthes* has often been confused and controversial. Although I am an ecologist rather than a taxonomist, a knowledge of the taxonomy of *Nepenthes* has proved very useful in my ecological research, and has recently developed into a strong secondary interest. An important point to bear in mind about current taxonomic trends in *Nepenthes* is that several different scientists hold different and competing views on a number of taxa. These will not be discussed in detail here, but various references are available to those who wish to delve into these issues in greater detail. Suffice to say that the interpretations followed here are my own (see Clarke 1997, 2001).

Brunei I spent most of 1989 and 1990 in Brunei performing ecological research on the animal communities of several lowland *Nepenthes* species. My primary interest was in the dynamics of insect communities found in the pitchers. I investigated whether the structure of these communities was in any way related to external habitat factors, such as geographical location, host pitcher structure and longevity, of habitat. In a preliminary survey, the communities of six lowland species were examined; *N. albomarginata*, *N. ampullaria*, *N. bicalcarata*, *N. gracilis*, *N. mirabilis* and *N. rafflesiana* (Clarke and Kitching, 1993). The food webs for two of these communities are shown in Fig. 1.

We concluded that the structure and dynamics of the food webs is related to the structure and longevity of the host pitcher species (see also Clarke, 1997), the number of sympatric *Nepenthes* species and various biogreographical factors. This views were largely in accordance with the findings of Beaver (1979, 1983, 1985), who studied *Nepenthes* communities in Peninsular Malaysia. However, recent research in Singapore, Malaysia and Sumatra (Kato *et al.*, 1993; Mogi and Chan, 1996, 1997; Sota *et al.*, 1998) contradicts our findings, and suggests that random colonisation processes and interspecific competition among the infaunal species are the primary determinants of community structure in *Nepenthes*. More recent studies (e.g., Clarke, 1998b; Cresswell, 2000) suggest that both views are tenable, and it seems apparent that a more holistic approach to future surveys is needed to decide which factors are the most important determinants of food web structure in the infaunal communities of *Nepenthes* pitchers.

My research into food web dynamics was sidetracked for a considerable period while I investigated the relationship between *N. bicalcarata* and a remarkable species of ant – *Camponotus schmitzi* (Clarke and Kitching, 1995). The ants bore holes into the hollow tendrils of *N. bicalcarata* pitchers, in which they nest. To feed, the ants move into the pitchers themselves, swimming in the fluid and removing large items of prey caught by the pitchers. This behaviour seems to be beneficial to the plants, as the contents of *N. bicalacarata* pitchers frequently become putrid if excess prey is caught. This often kills the infauna, which seems to be an important component of the pitcher's digestive system. In pitchers which contain very little prey, the ants sometimes feed directly upon the infauna. The infauna is of little use to the plant if the pitchers contain no prey, so again, the ants behaviour does not appear to have detrimental effects on the plant. It therefore appears that the ants represent the top predator in the *N. bicalcarata* food chain, and that the food web for this species is the most complex of any *Nepenthes* species studied to date (Clarke, 1998a, c)(Fig. 1).

In return for the benefits provided by the ants to the plant, they receive a food source and domicile, so the association can be considered to be a mutualistic one. Whether the relationship is obligate or facultative for either species is difficult to determine at present. Recenet research by Merbach et al. (2000, pers. comm.) has also shown that the thorns of *N. bicalcarata* pitchers contain giant nectaries, and that these are located in such a position that it is very difficult for most organisms to reach them. However, *C. schmitzi* ants are readily able to reach this nectar source, so it seems that in addition to feeding on the prey caught by the pitchers, the ants obtain nectar from the glands at the tips of the pitcher thorns.

***Nepenthes* of Borneo** At the conclusion of my field studies in Brunei, I commenced work on a more ambitious project – a textbook designed to cover all of the *Nepenthes* species of Borneo. This was based mainly on expeditions made during my time in Brunei, supplemented by extra trips to observe species in other parts of the island.

The highlight of this project was a series of expeditions to Kinabalu Park in Sabah during 1996. In addition to exploring Mount Kinabalu, I was able to visit Marai Parai and Mount Tambuyukon, home to *N. edwardsiana* – still the most spectacular of all *Nepenthes* as far as I am concerned. Several interesting observations were made on this expedition. We confirmed that *N. villosa* and *N.* ×*kinabaluensis* found on Mt. Tambuyukon in addition to Mt. Kinabalu (Clarke, 1998d). We found an immature plant of a new natural hybrid: *N. burbidgeae* × *N. edwardsiana*. Unfortunately, the plant was too small to photograph, but from the parentage it is clear that a mature plant of this cross could produce outstanding pitchers. Perhaps the most common species of *Nepenthes* on the summit of Mt. Tambuyukon is *N. rajah*. Here it grows in stunted, windswept health-like vegetation. The climate is extremely harsh and it appears that this species can tolerate much more severe conditions that those it is usually associated with.

The completion of *Nepenthes of Borneo* was a personal milestone and the fact that the book was generally well-received was very gratifying, but I soon came to the realisation that I wanted to do more. Taxonomic disputes relating to several Bornean taxa can only be resolved by undertaking field studies that lie beyond the limits of my enthusiasm, while further progress in ecological research requires additional long periods of field work, which I no longer have sufficient time for. I was approached to write a new text on the entire genus of *Nepenthes*, but I declined, as I do not think that I could do the project justice – it would take at least a decade to do the basic field work, and the financial resources required would be enormous. Instead, I made a counter-proposal to write a second book on the species from Sumatra and Peninsular Malaysia, and this kept me busy from 1997 to 2001.

Nepenthes benstonei In the course of researching *Nepenthes of Sumatra & Peninsular Malaysia* my attention was brought to a taxon from Kelantan that had not been described. Jebb & Cheek (1997) discussed it under *N. sanguinea*, but noted that it could belong to an undescribed species. B. Salmon (pers. comm.) observed the plant in the wild, and sent me several photos. Like Jebb & Cheek, I felt that this taxon could represent a new species, so while attending a Flora Malesiana conference in Kuala Lumpur in 1998, arranged for permission to collect specimens for the herbarium in

Kuala Lumpur. My investigations of this material led me to conclude that it was a distinct species, and I named it *N. benstonei*, after the late Benjamin Stone, who was the first to collect it (Clarke, 1999). This was the first species of *Nepenthes* that I described, and while I had never thought this would be very important, I was actually quite excited by it all!

***Nepenthes* of Sumatra and Peninsular Malaysia** This book was by far the most ambitious project I have every undertaken. Fortunately, unlike Borneo, it is possible to observe most of Sumatra's *Nepenthes* species in the wild with relative ease. Moreover, the taxonomic controversies relating to the Sumatran species are nowhere near as problematic as the Bornean ones. Therefore, with the kind assistance of several friends and colleagues, I was able to obtain photographs of every Sumatran *Nepenthes* species described to date. Of the 29 species I discussed, I was able to observe 27 of them in the field myself.

Many field sites were observed, and this time I devoted much more time to the study of herbarium collections. This was necessary because the emphasis of this text was on taxonomy as much as ecology. The highlight was the discovery of two new taxa of *Nepenthes*. One of these was described in the book as *N. jacquelineae*.

The completion of this project brought to an end all of my current research projects involving *Nepenthes*. It is now my intention to withdraw from active research on *Nepenthes* until I return to Australia, after which time I intend to resume ecological studies on food web dynamics, with the focus on *N. mirabilis* in Australia, and perhaps the species from New Guinea. The objective of listing these research highlights is, more than anything, intended to illustrate how enjoyable my work has been, and how privileged I feel to have been able to work in so many wonderful places, with such amazing plants and animals.

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Table 1. List of metazoan taxa collected from *N. bicalcarata* pitchers in Brunei by Clarke (1998a).

The ecological groupings are based on those of Beaver (1983), where B = nepenthebionts, P = nepenthephiles and X = nepenthexenes.

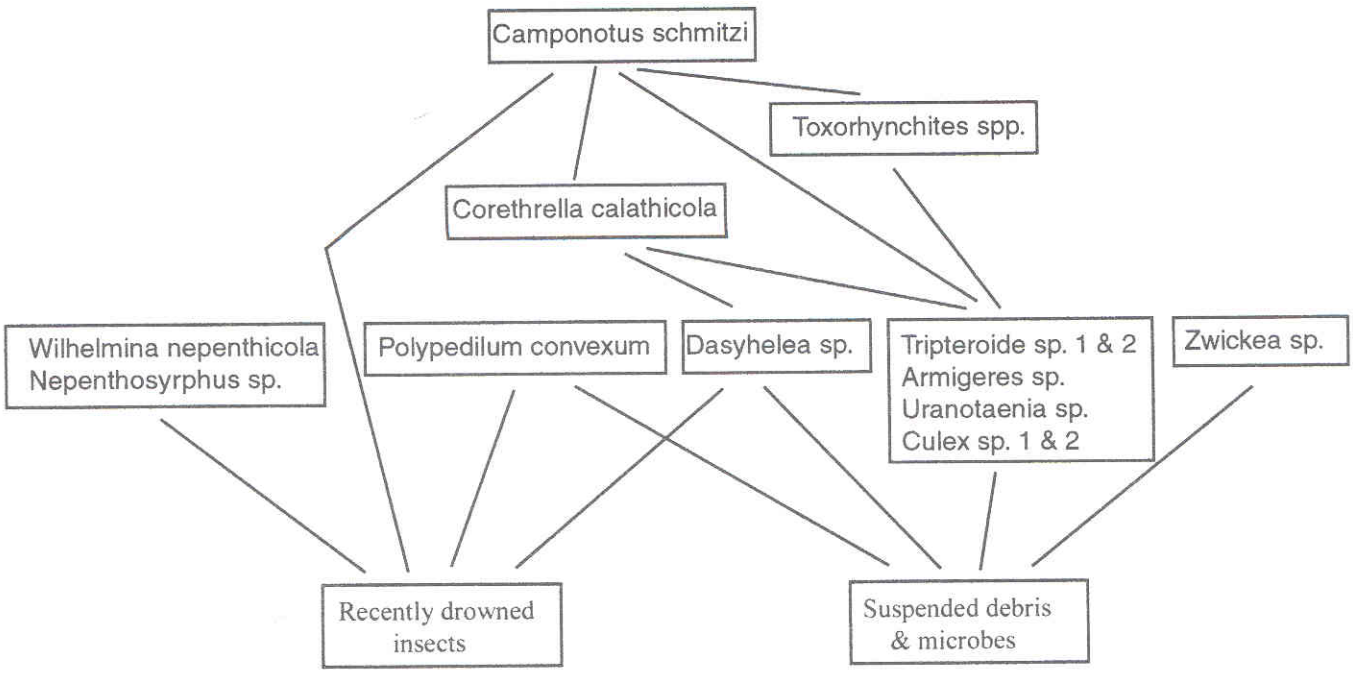
	Taxon	Family	Feeding strategy	Ecological Group
	Diptera			
1	*Polypedilum* (*Pentapedilum*) *convexum* Johannsen	Chironomidae	Detritivore	P
2	*Dasyhelea nepenthicola* Wirth & Beaver	Ceratopogonidae	Detritivore	B
3	*Aedes* (*Alanstonea*) *brevitibia* Edwards*	Culicidae	Detritivore	B?
4	*Armigeres* (*Armigeres*) sp.	Culicidae	Detritivore?	B?
5	*Culex* (*Lophocearomyia*) *coerulescens* Edwards	Culicidae	Detritivore	B
6	*Culex* (*L.*) *navalis* Edwards	Culicidae	Detritivore	B
7	*Culex* (*L.*) *hewetti* Edwards	Culicidae	Detritivore	B
8	*Culex* (*L.*) *eminentia* Leicester*	Culicidae	Detritivore	B
9	*Toxorhynchites* (*Toxorhynchites*) sp. 1	Culicidae	Predator	B?
10	*Toxorhynchites* (*Toxorhynchites*) sp. 2	Culicidae	Predator	B?
11	*Toxorhynchites* (*Toxorhynchites*) sp. 3	Culicidae	Predator	B?
12	*Tripteroides* (*Rachionotomyia*) *nepenthis* Edwards	Culicidae	Detritivore	B
13	*Tripteroides* (*R.*) *nepenthesimilis* Mattingly*	Culicidae	Detritivore	B
14	*Tripterioides* (*R.*) *tenax* Meijere*	Culicidae	Detritivore	B
15	*Uranotaenia* (*Pseudoficalbia*) *moultoni* Edwards	Culicidae	Detritivore	B
16	*Corethrella calathicola* Edwards	Chaoboridae	Predator	B
17	*Corethrella* sp. 1	Chaoboridae	Predator	X?
18	*Lestodiplosis* sp.	Cecidomyiidae	Predator	B
19	*Xenoplatyura* sp.	Mycetophilidae	Predator	B

20	*Megaselia campylonympha* Schmitz	Phoridae	Detritivore	B
21	*Nepenthosyrphus oudemansi* Meijere*	Syrphidae	Detritivore	B
22	*Wilhelmina nepenthicola* Villeneuve	Calliphoridae	Detritivore & predator	B
23	*Nepenthomyia malayana* Kurahashi & Beaver	Calliphoridae	Detritovore & predator	B
24	Calliphorid sp. 1	Calliphoridae	Detritovore & predator	B?
	Lepidoptera			
25	*Eublemma radda* Swinhoe†	Noctuidae	Herbivore	B
26	Lepidopteran sp.	?	Herbivore	B
	Hymenoptera			
27	*Camponotus* (*Colobopsis*) *schmitzi* Schuitemaker & Stä rke	Formicidae	Detritivore & predator	B
28	Encyrtid sp.	Encyrtidae	Pupal parasitoid	B
	Aranae			
29	*Misumenops nepenthicola* Pocock	Thomisidae	Predator	B
	Acari			
30	*Zwickea nepenthesiana* Hirst	Anoetidae	Detritivore	B
	Brachyura			
31	*Geosesarma* sp.	Grapsidae	Detritivore	P?
	Anura			
32	*Philautus* sp.	?	N/A	P?
33	? sp.	?	Detritivore	P?

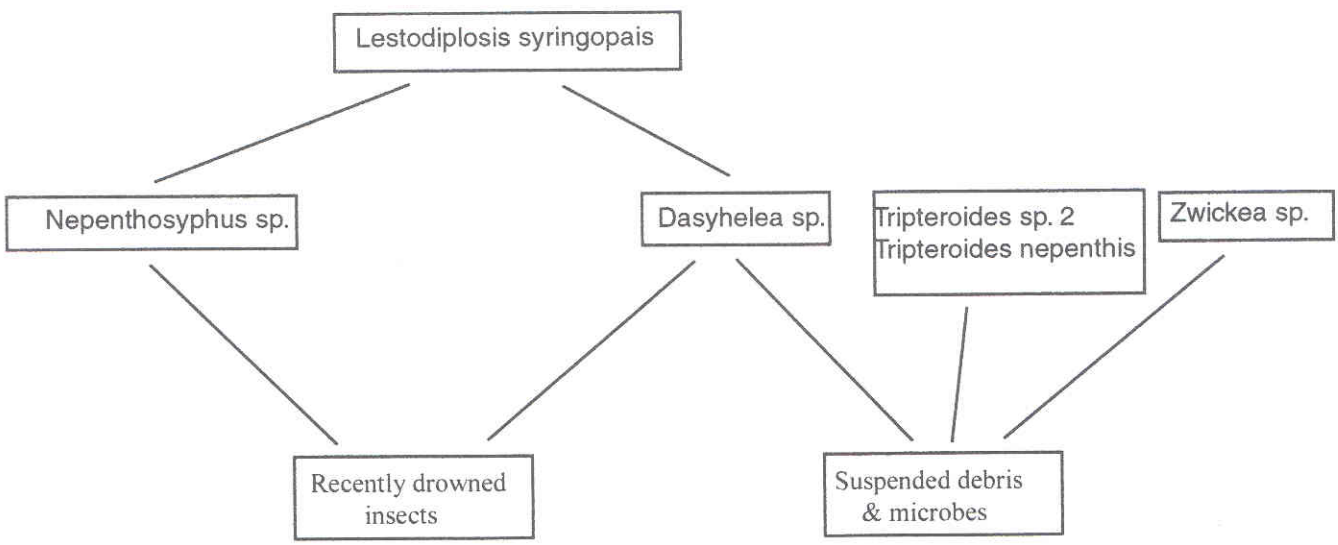
* Denotes species which were not identified with certainty.

† Denotes species attributed to *N. bicalcarata* by Beaver (1983) which were not found in *N. bicalcarata* pitchers in this study.

Figure 1. Food web diagrams for the metazoan communities of two Nepenthes species from Brunei (adapted from Clarke & Kitching (1993)). Arrows between species indicate feeding interactions.



(a) Nepenthes bicalcarata



(b) Nepenthes rafflesiana

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Phylogeny of the sundews, *Drosera* (Droseraceae) based on chloroplast *rbcL* and nuclear 18S ribosomal DNA Sequences

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Abstract. *Drosera* includes nearly 150 species distributed mainly in Australia, Africa, and South America, with some Northern Hemisphere species. In addition to confused intrageneric classification of *Drosera*, the intergeneric relationships among the *Drosera* and two other genera in the Droseraceae, *Dionaea* and *Aldrovanda*, are problematic. We conducted phylogenetic analyses of DNA sequences of the chloroplast *rbcL* gene for 59 species of *Drosera* covering all sections except one. These analyses revealed that 5 of 11 sections including 3 monotypic sections are polyphyletic. Combined *rbcL* and 18S rDNA sequence data revealed that all *Drosera* species form a clade sister to a clade including *Dionaea* and *Aldrovanda*. MacClade reconstructions indicated that aneuploidy occurred several times in a Australian clade, while the chromosome numbers in the other clades are mostly stable. *D. regia* and most Australian species were clustered basally, suggesting that *Drosera* originated in Africa or Australia. The *rbcL* tree indicates that Australian species expanded their distribution to South America, and then to Africa. Expansion of distribution to the North Hemisphere from the South Hemispere occurred in a few different lineages.

Introduction

The genus *Drosera* includes nearly 150, mostly perennial, species (Juniper *et al.*, 1989; Lowrie, 1998). Although *Drosera* has a worldwide distribution, the vast majority of species are found in the Southern Hemisphere, especially in Southwestern Australia. *Drosera* have active flypaper traps and capture their prey with mobile glandular hairs that are present on the adaxial leaf surface. *A. vesiculosa* and *Di. muscipula* share a similar trapping mechanism, called a snap trap, exclusive to these two taxa (Juniper *et al.*, 1989). *A. vesiculosa* is a floating aquatic species that is found throughout the Old World and Northern and Eastern Australia, while *Di. muscipula* is a terrestrial plant that is endemic to marshy habitats on the coastal plains of North and South Carolina (Juniper *et al.*, 1989). Relationships among *Drosera*, *Aldrovanda*, and *Dionaea* have not been solved with high statistical confidence in either *rbcL* or *matK* trees, although the monophyly of *Drosera*, *Dionaea*, and *Aldrovanda* is widely accepted, based on the morphological and molecular data (Williams *et al.*, 1994; Meimberg *et al.*, 2000).

New systems on *Drosera* have been proposed recently (Marchant *et al.*, 1982; Seine and Barthlott, 1994; Schlauer, 1996), the delimitations of the subgenera and sections of *Drosera* are controversial. Williams *et al.* (1994) inferred the phylogenetic relationship of 12 *Drosera* species covering most sections *sensu* Sein and Barthlott (1994), and further analyses with more taxa are obviously necessary to overview the phylogeny of *Drosera*, which is morphologically divergent and includes more than 150 species.

In this presentation, we inferred the intergeneric relationship among *Drosera*, *Aldrovanda*, and *Dionaea*, and Interspecific relationships among the genus *Drosera* using *rbcL* and 18S rDNA. Base d on the inferred phylogenetic tree, evolution of chromosome number and biogeography of *Drosera* was discussed.

Materials and Methods

All subgenera and sections of *Drosera sensu* Seine and Barthlott (1994) except sect. *Meristocaulis*, *Dionaea*, and *Aldrovanda* were used in this study. Total DNA extraction, sequencing, and phylogenetic analyses generally followed Hasebe *et al.* (1994).

Results and Discussion

Parsimony analysis produced the 4608 most parsimonious (MP) trees of 1087 steps in 12 islands (Maddison 1991) using the data matrix of 1227 bp *rbcL* for the 75 taxa including 16 outgroup. A strict consensus tree of the 4620 MP trees is shown in Fig. 1 with bootstrap values. The 1648 bp region of 18S rDNAs and the 1227 bp *rbcL* of *Dionaea, Aldrovanda,* and some representative species of *Drosera* were used to infer their phylogenetic relationship. Parsimony analysis produced a single MP tree of 589 steps (Fig. 2)

The MP tree for the combined dataset in Fig. 2 showed that *Dionaea* and *Aldrovanda* form a sister group with 80% BP. This result indicates that the flypaper system of *Drosera* and the snap trap system of *Dionaea* and *Aldrovanda* were established early in the evolution of these carnivorous plant taxa, but it was not possible to elucidate which trap system the common ancestor of these two lineages had or whether these two systems evolved independently from non-carnivorous plants. The sister relationship of *Dionaea* and *Aldrovanda* indicates a single evolutionary origin of the elaborate snap trap system in plants, although terrestrial *Dionaea* and aquatic *Aldrovanda* have different habitats.

The *rbcL* tree is not concordant with any intrageneric classification of *Drosera*, although some clades characterized by morphological characters, chromosome number, and geographic distribution were detected in the *rbcL* tree. It is necessary to revise the classification of *Drosera* by incorporating the *rbcL* tree data and further analyses of morphological characters.

Our analysis showed that conspicuous chromosome diversity caused by both aneuploidization and polyploidization is observed extensively in the clade from *D. stolonifera* to *D. glanduligera*, which is almost exclusively Australian, while chromosome number is moderately conserved in the other clades.

D. regia is basal, while the clade including all the other African species except *D. indica* clustered at the terminal position. *D. arcturi*, which is native to Australia and New Zealand, is also basal, and all the other Australian species clustered next to *D. regia* and *D. arcturi*, indicating that the origin of *Drosera* was in Africa or Australia. The *rbcL* tree shows that the South American species arose by dispersal from Australia, and that the African species other than *D. regia* and *D. indica* arose from South America. Dispersal from Australia to South America also likely occurred in the clade that includes *D. brumannii* and *D. sessilifolia*. Dispersal from Australia to Asia via Southeast Asia occurred in *D. burmannii*, *D. indica*, and *D. peltata*, although it is not known why these species were the only members of their respective clades to expand their distributions in such a manner. Smaller numbers of Drosera species are distributed in the Northern Hemisphere than in the Southern Hemisphere, as mentioned above. Our analysis suggests that all the Northern Hemisphere species examined (*D. rotundifolia*, *D. anglica*, *D. filiformis*, *D. capillaries*, *D. brevifolia*, *D. indica*, *D. burmannii*, and *D.*

peltata) expanded their distributions from the Southern Hemisphere.

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Figure legends

Figure 1. A strict consensus tree of the 4620 most parsimonious trees of *rbcL* sequences. Bootstrap values are indicated above the branches occurring in more than 50% of 10,000 bootstrap replicates. The higher classifications *sensu* Schlauer (1996) and Seine and Barthlott (1994) are shown on the right.

Figure 2. The most parsimonious tree resulting from parsimony analysis of the combined *rbcL* and 18S rDNA sequences. The numbers above the branches are the bootstrap values greater than 50% for 10,000 bootstrap replicates.

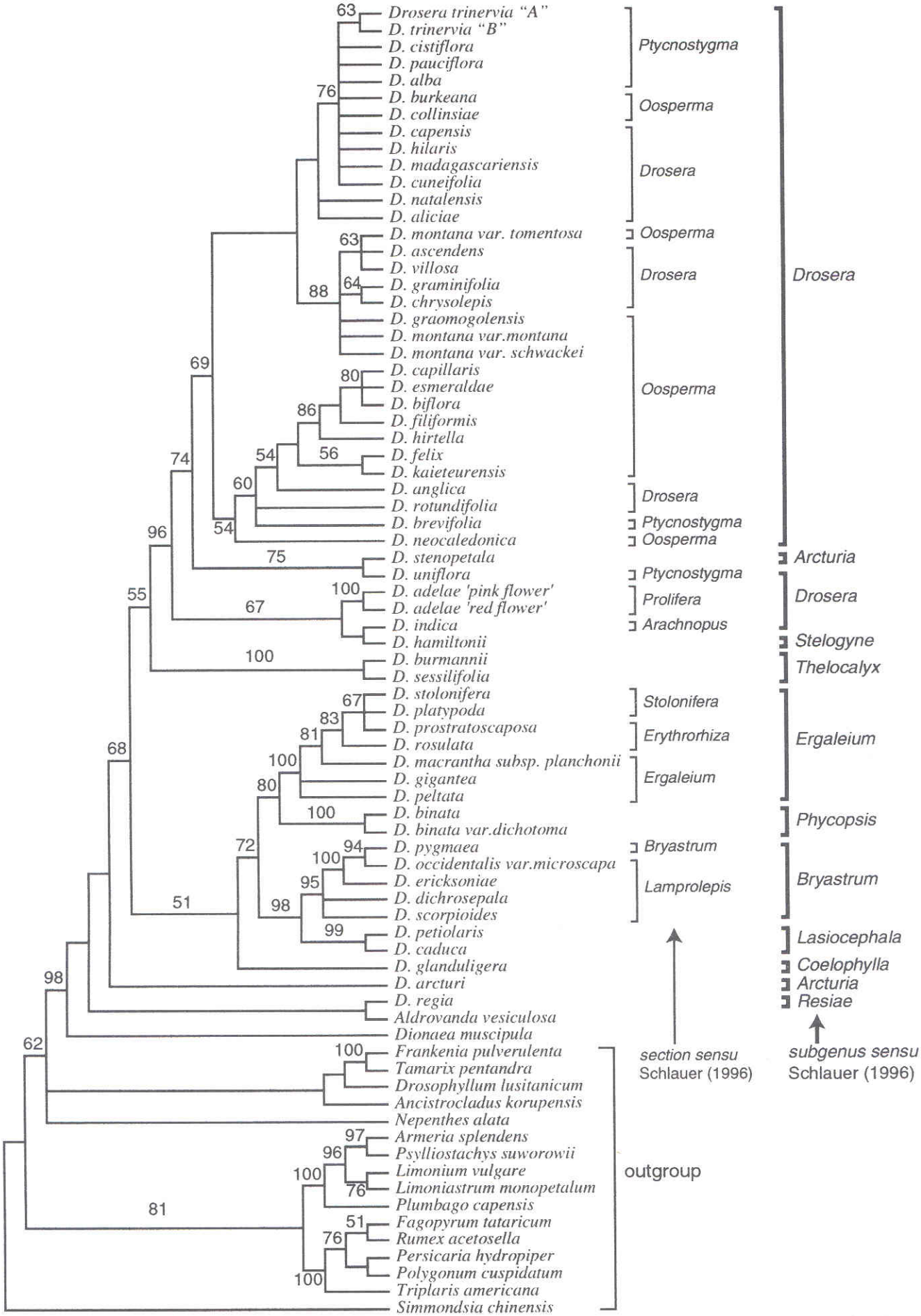

Figure 1 A strict consensus tree of the 4620 most parsimonious trees of *rbcL* sequences. Bootstrap values are indicated above the branches occurring in more than 50% of 10,000 bootstrap replicates. The higher classifications *sensu* Schlauer (1996) and Seine and Barthlott (1994) are shown on the right.

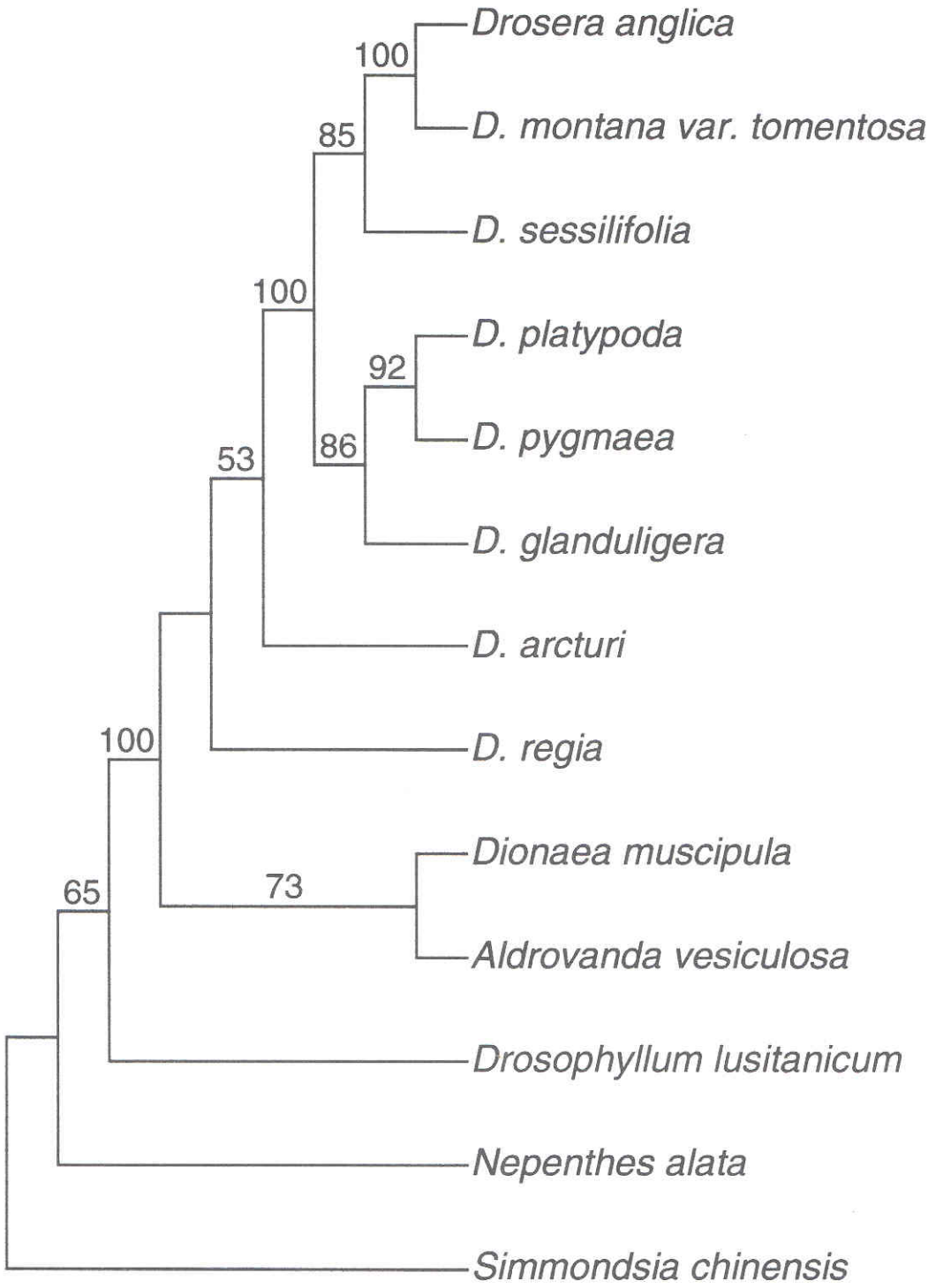

Figure 2 The most parsimonious tree resulting from parsimony analysis of the combined *rbcL* and 18S rDNA sequences. The numbers above the branches are the bootstrap values greater than 50% for 10,000 bootstrap replicates

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Population structure of *Nepenthes* species (pitcher plants) from Weston, Sipitang in Sabah

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Abstract. *Nepenthes ampullaria, N. rafflesiana* and *N. albomarginata* were recorded in the two study plots from Weston.. A total of 112 pitcher plants were recorded from these plots, equalling an area of 0.04 hectare. Of these, 52 belonged to *N. albomargianata*, followed by *N. rafflesiana and N. ampullaria* with 40 and 20 plants respectively. These species showed a very strong tendency to grow in forest gap. The I_d values greater than 1 of these three species showed contagious dispersion pattern. The χ^2 test indicated that the dispersion pattern of all species was significantly different from random dispersion. Size class distribution and population structure showed variation for both species. There were more seedlings and juveniles than mature plants in all species thus indicating regenerating populations. The number of leaves bearing pitchers is variable between size classes within the species. The χ^2 test showed significant and no differences of pitcher production between size in *N. albomarginata* and *N. rafflesiana* respectively

Introduction

Pitcher plant is potentially capable of reproducing new individuals either by vegetative shoots (asexual mode) or seeds (sexual mode). The seedling has a rosette of leaves spreading on all sides of a suppressed stem and very short inter node, and each leaf normally bears a pitcher at its tip. These rosette stage may remain for two or three years (Green, 1967); only when the stem become erect does it begin to grow rapidly in length (Holttum, 1954) and in most species the inter nodes gradually lengthen with increasing age attaining 20-30 cm in length when the plant is upright. During seedling and juvenile stages the plants produce ground pitchers and they were sterile. The stem then elongates continuously and, with the support of strong curled tendrils, it may climb up trees in primary forest (heath forest), or on to *Dicranopteris linearis* (ferns) or shrubby *Melastoma malabathricum* (senduduk) in degraded and secondary vegetation. This phase of life is called mature pitcher plant.

Mature pitcher plants can produce both ground and aerial pitchers which depending on species are similar or different in shape. Examples of pitcher plants capable of growing in tall canopy forest, in open habitats, and shrubby vegetation include *Nepenthes ampullaria, Nepenthes bicalcarata, Nepenthes macrovulgaris, Nepenthes gracilis* and *Nepenthes rafflesiana*. These species climb up to the tree canopy top preceding flowering. In open area, species, such as *Nepenthes ampullaria* may produce flower when the plant is a few meters tall but growing to 30 m tall in dipterocarp forest before flowering (Adam *et al.*, 1994; Adam, 1995) . If the stem fails to find the support, the stem lies on the ground, rooting abundantly at the nodes, and producing short lateral branches with dense clusters of leaves with reduced laminas and large pitchers (Macfarlane, 1908). The switch from the lower to upper pitcher type appears usually to coincide with the onset of flowering (Juniper *et al.*, 1989).

The objectives of this research were to determine species composition of *Nepenthes* present; secondly, to determine population density and mapping of every species identified; thirdly, to identify distribution population pattern, size class distribution; and fourthly, to determine the relationship of pitcher production with stem length.

Materials and Methods

The study on the species heterogeneity, and population structure of *Nepenthes* from heath forest in Weston (30 m altitude), Sipitang District in Sabah was carried out.

In order to accomplish these objectives, two plots each measuring 10 m x 20 m was set up. These plots were each subdivided into 25 subplots, each measuring 2 m x 4 m respectively. The division of the larger plots were necessary in order to determine the spatial pattern of arrangement of individuals in the population, also known as the population dispersion pattern (Brower and Zar, 1977). The analysis of these data using Morista's Index of Dispersion (I_d) and the Chi-square (χ^2) test requires this division.

All individual plants of *Nepenthes* in these plots were identified to species in the field and mapped to scale. Every plant mapped was numbered using plastic tag. Stem length, number of leaves without pitcher and number of leaves bearing pitchers were measured and countered. The life stage of each plant was recorded namely, the seedling (Size Class 1 – SC1), juvenile stage, the upright plant capable of producing ground pitchers (SC2-SC3), the mature sterile which produce upper and lower pitchers but not producing flowers (SC4-SC8), and mature fertile male and female plants. This arbitrary classification is necessary to avoid confusion, to determine the size class distribution of stem, and to ease enumeration of the plants found in the plots.

The dispersion pattern of *Nepenthes* plants was computed using I_d, and the departure of an observed dispersion pattern from randomness was assessed statistically using χ^2 test (Brower and Zar, 1977). To established the relationship of percentage frequency of leaves producing pitchers with size, it was necessary to classified the stem length into four size classes: SC1=0.1-40 cm; SC2 = 40.1-80 cm; SC3= 80.1-120 cm; SC4 = > 120 cm. The χ^2 test in contingency table was used in analyzing the percentage frequency of leaves producing pitchers in relation to these four size classes.

Results and Discussion

Three *Nepenthes* species were present from the two study plots from Weston, Sipitang District in Sabah. These species were *Nepenthes ampullaria,, N. albomargnata* and *N. rafflesiana*.

A total of 112 pitcher plants were enumerated from the two study plots. Of these, 52 plants belong to *N. albomarginata*, 40 plants to *N. rafflesiana*, whereas 20 plants belong to *N. ampullaria*. These species were confined in well-defined patches, growing within the forest gap of high canopy dipterocarp forest (Fig. 1) and gap of the heath forest. (Fig. 2).

The spatial arrangement of *N. ampullaria* in plot 1 was illustrated in Fig. 1, whereas *N. albomarginata* and *N. rafflesiana* plants illustrated in Figure 2. The dispersion pattern of *N. ampullaria* in plot 1 was contagious (Fig. 1) with I_d value of 4.8; whereas the dispersion pattern of *N. albomarginata* and *N. rafflesiana* were also contagious (Figure 2) with I_d values of 3.4 and 2.9 respectively; and the χ^2 values of *N. ampullaria, N. albomarginata* and *N. rafflesiana* (Table 1) calculated were greater than X^2 0.05, 24= 36.4. These species have a very strong tendency to grow in forest gaps thus indicating light may be required for their normal growth.

In a regenerating population, larger numbers of plants fall within the small size classes, with fewer bigger plants representing successive mortality, as the plants grow older. In *N. albomargianata*, 44 of the plants in the population of plot 2 belong to SC1-4 and the other 10 plants fall into SC5-8. Similarly, 36 plants of *N. rafflesiana* fall into SC1-4 and the other 4 plants fall into SC5-8 (Table 2). However in *N. rafflesiana,* nine of the total so mature sterile plants belong to SC1-4. Regression analysis showed a significant decrease in the distribution frequency of pitcher plants density in relation to increasing size in *N. albomarginata* (Y= -0.0007x + 3.09; Sign. F = 0.006) but not significant in *N. rafflesiana* (Y=-0.009x + 4.86; Sign. F = 2.76); *N. ampullaria* (Y=-0.003x + 4.44: Sign. F=0.21).

Nepenthes population comprise of individual plants of different life stages. In this study, four life stages are recognized namely seedling (SC1), juvenile (SC2 and SC3), mature sterile (SC4-SC8) mature fertile plant (Table 2). *Nepenthes rafflesiana* and *N. albomargianata* populations have relatively higher density of seedlings (SC1) and no mature fertile plants in *N. ampullaria* and *N. rafflesiana.* A total of two male and three female plants bearing inflorescence are recorded in *N. albomargianata.* The absence of plants producing either male or female flowers may be due to the failure of the mature sterile plants to reach above the canopy. The absence of vegetative shoots in these two species indicates that vegetative reproduction is not operative in either species.

A total of 1011 add 392 leaves of *N. albomarginata, N. amplularia* and *N. rafflesiana* were sampled (Table 3). The number or percentage of leaves bearing pitchers is variable between the four size classes within the species. The χ^2 test reveal significant differences of pitcher production between size in *N. albomarginata* (χ^2=37.9> $X^2_{0.05\ \&\ 0.01,\ 3}$=7.815 & 11.34; Table 3) but no significant difference in *N. rafflesiana*. Differences between species in frequency of pitcher production in the study plot suggesting intrinsic causes.

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Table 1. The I_d, χ^2 goodness of fit test, density and dispersion pattern of *N.ampullaria, N. rafflesiana* and *N. albomarginata* in 2 sampling plots from Weston, Sipitang in Sabah

*P	Species	SP	PS (m)	I_d	X^2	$\chi^2_{0.05, 24}$	D	DP
1	*N. ampullaria*	25	10 x 20	4.8	120	36.4	20	C
2	*N. rafflesiana*	25	10 x 20	2.9	111	36.4	40	C
2	*N. albomarginata*	25	10 x 20	3.4	180	36.4	52	C

***P: All the plots was located at 30 m altitude; PS Size of all plot sampled was 10 x 20 m; SP= All plots were divided into 25 subplots (SP), each with 2 m x 4 m in size; D = Density; DP = Dispersion pattern.**

Table 2. Size class distribution of *N.ampullaria, N. rafflesiana* and *N. albomarginata* in 2 sampling plots from Weston, Sipitang, Sabah.

Plot	Sp.		SC 1	SC 2	SC 3	SC 4	SC 5	SC 6	SC 7	SC 8	STR	MP	FP	Σ
1	*NM*	No.	2	7	0	0	4	0	4	3	20	0	0	20
		%	10	35	0	0	20	0	20	15	100	0	0	100
2	*NR*	No.	16	10	7	3	1	1	0	2	40	0	0	40
		%	26.8	23	16.1	6.9	2.3	2.3	0	4.6	100	0	0	100
2	*NA*	No.	33	10	0	1	1	0	0	0	45	2	7	52
		%	62.7	22	0	1.9	1.9	0	0	0	83.9	3.8	13.3	100

Plot; Sp.- Species; STR- Sterile plant; MP- Male plant; FP- Female plant; SC- Size class; SC1:10.1-20 cm (seedling); SC2: 20.1-40 (Juvenile); SC3: 40.1-60 cm (Juvenile); SC4: 60.1-100 cm (mature sterile); SC5: 100.1-140 cm (mature sterile); SC6: 140.1-180 cm (mature sterile); SC7: 180-250 cm (mature sterile); SC8: >250 cm (mature sterile); No.- Number of plants; %- percentage; *NM- N.ampullaria; NR – N. rafflesiana, NA – N. albomarginata.*

Table 3. The $\chi 2$ goodness of fit test in contingency tables of percentage mean frequency of pitcher production in relation to stem length (SC) of *Nepenthes rafflesiana* and *Nepenthes albomarginata* from Weston in Sipitang

	Species	SC1 0-40 cm	SC2 40.1-80 cm	SC3 80.1-120 cm	SC4 >120 cm	%UFPP	χ^2	N
1	*Nepenthes. rafflesiana*	28.6 NS	32 NS	28.6 NS	21.7 NS	29 NS	1.29	392
2	*Nepenthes albomarginata*	39.0 NS	*22.8**	*11.7**	33.6 NS	37.1***	11.4	1001

χ^2 =7.81, Degree of freedom = 3; P=0.05

χ^2 =11.34, Degree of freedom = 3; P=0.01

*Significant different from percentage mean frequency of pitcher production (% UFPP) at P=0.05

*** Not Significant different from percentage mean frequency of pitcher production (% UFPP) at P=0.01

NS Not significant from % UFPP at P=0.01 and 0.05

Italic values of % are significantly different from %UFPP

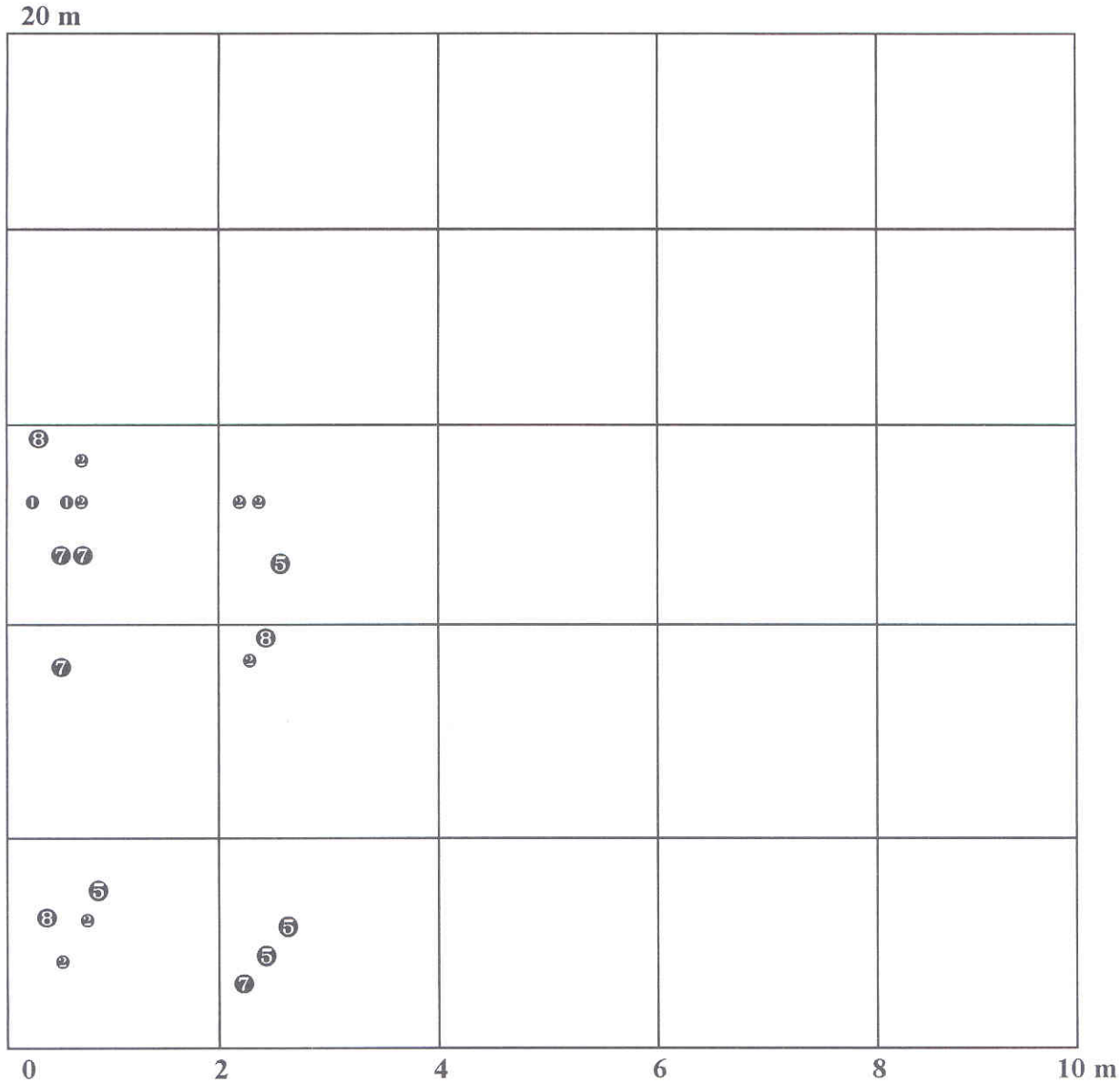

Figure 1. Contagious dispersion of *N. ampullaria* in P1 (30 m) growing in gap of high canopy lowland Dipterocarp forest in Weston, Sabah

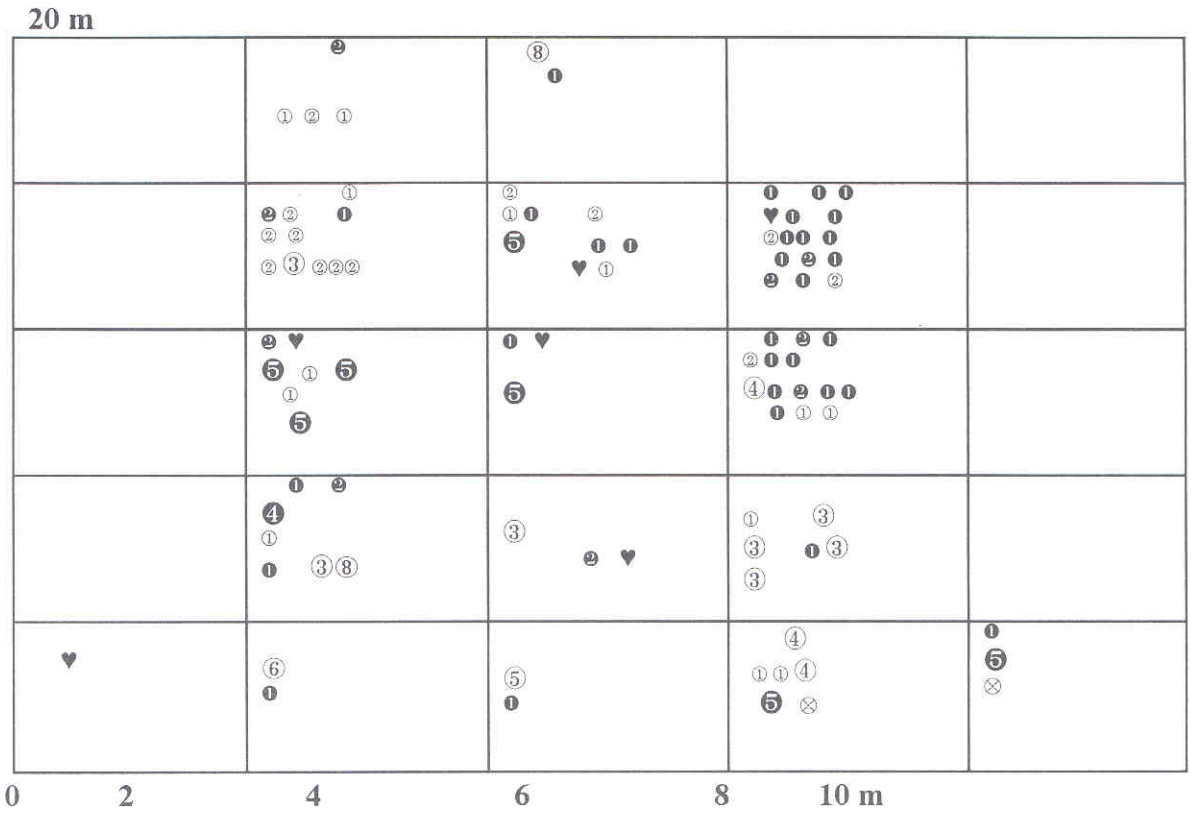

Figure 2. Contagious dispersion of *N. albomarginata* (●⊗♥) & *N. rafflesiana* (○✣▲) in P2 (30 m) growingg in gaps of heath forest in Weston, Sabah.

Key to Size class (Fig. 1-3)

❶①: 1- 10 cm
❷②: 10.1-40 cm
❸③: 40.1-60 cm
❹④: 60.1-100 cm
❺⑤: 100.1-140 cm
❻⑥ 140.1-180 cm
❼⑦: 180.1-250 cm
❽⑧: 250.1-500 cm
⊗: **Male plant bearing male inflorescence**
♥: **Female plant bearing female inflorescence**

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Pitcher plants of Sumatra and Borneo by video record (VIDEO)

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VIDEO shows *Nepenthes* of Sumatra and Borneo with much great information regarding the characteristics of (1) the distribution center; species diversity; diversity of pitcher morphology; and habitat diversity, and (2) insect capture system; nectar glands; and other characteristics seen in *Nepenthes bongso* on strange insect capture system, *N. bicalcarata* on slide of death, and *N. veitchii* on water dam adapted for epiphytic habitat, etc. VIDEO also exhibits that the pitcher is a micro-cosmos growing mosquitos, spiders, nematoda, ants, frogs and so on.

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Nepenthes species of the Hose Mountains in sarawak, Borneo

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Abstract. This report summarizes the observations made during several field trips to the Hose Mountains of Sarawak between October 2000 and December 2001. Eight species of Nepenthes were found in this region: *N. ephippiata* (a new record for Sarawak), *N. fusca, N. hirsuta, N. reinwardtiana, N. tentaculata, N. veitchii, N. sp. 'A'*, and *N. sp. 'B'*. The latter two species may represent new and undescribed taxa.

Introduction

The Hose Mountains is a remote mountain range located in central Sarawak between the watersheds of Sungai Baleh and Sungai Balui (see Figs. 1 and 2). The region has been poorly explored botanically in the past century largely due to difficulty of access. Because of its high altitude and relative isolation, this area was targeted for this project due the potentially high diversity of *Nepenthes* species occurring there.

Prior research at the Sarawak Herbarium revealed several unusual *Nepenthes* specimens which had been collected in the Hose Mountains. Most notably S. 19023 which had been collected on an expedition to Bukit Temedu in March 1964. This plant was labelled as *N. lowii*, but clearly showed differences from this species in the form of the pitcher. Another specimen, S. 21114 from Bukit Lumit, Ulu Sungai Mujong, which was labelled as *N. pilosa* showed some important differences with that species upon closer examination.

Topography within the Hose Mountains is extremely rugged, and this is largely why the region has remained untouched by logging activities. The range itself is composed of dacite and related rocks which frequently form vertical or near-vertical cliff faces. This formation is surrounded by foothills composed of sandstone, shale, mudstone, lignite, and siltstone.

The highest elevation in the range is Bukit Batu at 2028 meters and there are at least four other peaks which reach over 1800 meters. The nearest mountain range of a similar altitude is Bukit Batu Tiban (elevation 1920 meters) situated on the Sarawak-Kalimantan border. Between these two ranges stretches approximately 110 km of lower elevation forest not exceeding 1300 meters in elevation, and generally below 800 m. Because of this, the Hose Mountains represent a relatively isolated range, and therefore would have a high potential for endemism of montane plant species.

Materials and Methods

A series of field trips were made to the Hose Mountain region between October 2000 and June 2001. During each trip photographs were taken of plants and information recorded on habitat and altitudinal distribution. Collecting permits were obtained through the Sarawak

Biodiversity Centre and the Sarawak Forestry Department. This allowed for collection of seeds as well as voucher specimens for deposition at the Sarawak Herbarium.

In October 2000, a preliminary reconnaissance expedition was made to Gunung Gelanggang (Ulu Sungai Betun) at the southern end of the mountain range. This trip was unsuccessful in reaching the summit and was forced to retreat at 1200 m due to vertical cliff faces preventing further ascent.

In June 2001, a more fully equipped expedition was made to Bukit Batu (Ulu Sungai Mujong) in the northern end of the range. A base camp was established at 1200 m on the west flank of the mountain from which day excursions were launched. Here also vertical cliff faces along the ridge hindered ascent, making progress above 1400 m extremely difficult. The party reached a maximum elevation of 1700 m before retreat was necessary.

A follow up expedition to Bukit Batu in December 2001 was focused primarily on exploring vegetation on the lower slopes of the mountain. Several smaller summits (elevation less than 1400 m) which were easily accessed were explored in greater detail.

Results

In total, eight taxa of *Nepenthes* were found on the expeditions (see Table 1). These are summarized below:

N. ephippiata Danser

This species was found growing in mossy ridge top vegetation above 1600 m. Most plants were epiphytic, and their large pitchers were conspicuous in the short canopy. This represents a new record for Sarawak, since this species has only previously been recorded from Central Kalimantan. The plants differ from the more widespread *N. lowii* in having only short tubercles (rather than hair-like growths) and a more pronounced peristome. Unlike specimens of *N. ephippiata* collected from Central Kalimantan, these plants did not exhibit the decurrent petiole base. Plants observed in the wild correlated with specimen S. 19023 from the Sarawak Herbarium.

N. fusca Danser

This species was observed at numerous locations in the Hose Mountains at 700-1200 m. Plants occurred most commonly as epiphytes in large trees in lower montane forest, but were also occasionally found on roadside embankments where they grew among ferns and grasses. Plants were typical in form of the species, but exhibited some variation in color ranging from red to purple spotted lower pitchers, and spotted to pure orange upper pitchers.

N. hirsuta Hook. f.

Plants of this species were found growing in sub-montane sandy heath forest on a plateau at approximately 800 m, sympatric with *N. veitchii*. Most plants grew terrestrially in full shade, but some were observed growing in full sun on road embankments. Pitchers were pure light green sometimes with faint purple spotting.

N. reinwardtiana Miq.

This species was the only *Nepenthes* encountered below 800 m. Although primarily an epiphyte in lowland forest, it was found in great abundance along logging roads between 300 and 700 m where it grew in full sunlight. Pitcher coloration was most commonly green, but also occurred in red and purple forms.

N. tentaculata Hook. f.

This species was found to be common in mossy forest above 1500 m. Occasionally plants were observed growing imbedded in moss on shady vertical rock cliffs or beneath grass on steep slopes. Lower pitchers exhibited an unusual peristome in that the inner teeth were extended into a fine comb-like ridge. Otherwise plants were of a form typical for this species in Sarawak.

N. veitchii Hook. f.

Plants of this species were found growing epiphytically in sub-montane heath forest at an altitude of 800 m. Occasionally plants also occurred as terrestrials growing on road embankments up to 1200 m. Pitcher color was light green with yellow to red-striped peristomes.

N. sp. 'A'

This species was found growing terrestrially in mossy forest ridge top vegetation at an altitude above 1500 m. In general form, these plants were very similar to *N. pilosa*, with broad petiolate elliptic leaves and a dense indumentum. They differed in having broadly infundibuliform pitchers which lacked any appendages on the lower surface of the lid (unlike the hooked appendage found in *N. pilosa*). In addition, the stems, leaf bases, and pitchers were covered in a dense abundance of large black nectar glands. Pitcher color was pale green with light red spotting. Plants observed correlated well with specimen S. 21114 in the Sarawak Herbarium.

N. sp. 'B'

Plants of this species were observed growing on steep slopes of *Dipteris* and *Dicranopteris* ferns at an altitude of 900-1300 m. The unusual pitcher morphology made it apparent that this represents a new and undescribed taxa. The most unusual feature was the peristome of the upper pitchers which was flattened and very broad (to 3 cm wide), with no apparent ribs typical of other *Nepenthes*. Upper pitchers measured up to 15 cm in length and were broadly infundibular in shape. Lower pitchers resembled those of *N. fusca* but with a wider more flared peristome.

N. fusca x veitchii

A single plant of this hybrid was found growing on a roadside embankment at approximately 1000 m. Pitcher form was intermediate between the parent species.

N. sp. 'B' x fusca ?

Three plants representing what may be this hybrid were found growing on roadside embankments at approximately 1200 m.

Discussion

With eight recorded taxa, the Hose Mountains represents a very diverse region for *Nepenthes*. Two species, *N. sp. 'A'* and *N. sp. 'B'* are possible endemics to the mountain range. It can be assumed that the species observed during this field work occur throughout the Hose Mountains at similar habitats and elevations as those recorded. Montane species such as *N. tentaculata, N. sp. 'A',* and *N. ephippiata* likely occur at higher elevations than those reached by the expeditions (see Fig. 1).

Due to the lack of thorough exploration, there exists a high probability for additional *Nepenthes* species occurring in the Hose Mountains. Species such as *N. ampullaria, N. mirabilis, N. rafflesiana, N. gracilis* may occur on the lower slopes or in surrounding foothills. Further fieldwork on the summit of the mountains may reveal additional montane species such as *N. stenophylla* or new taxa.

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Table 1. Nepenthes taxa from the Hose Mountains

	Species
1	***N. ephippiata*** Danser
2	***N. fusca*** Danser
3	***N. hirsuta*** Hook. f.
4	***N. reinwardtiana*** Miq.
5	***N. tentaculata*** Hook. f.
6	***N. veitchii*** Hook. f.
7	***N. sp. 'A'***
8	***N. sp. 'B'***
	Hybrids
	N. fusca x veitchii
	N. fusca x sp. 'B' ?

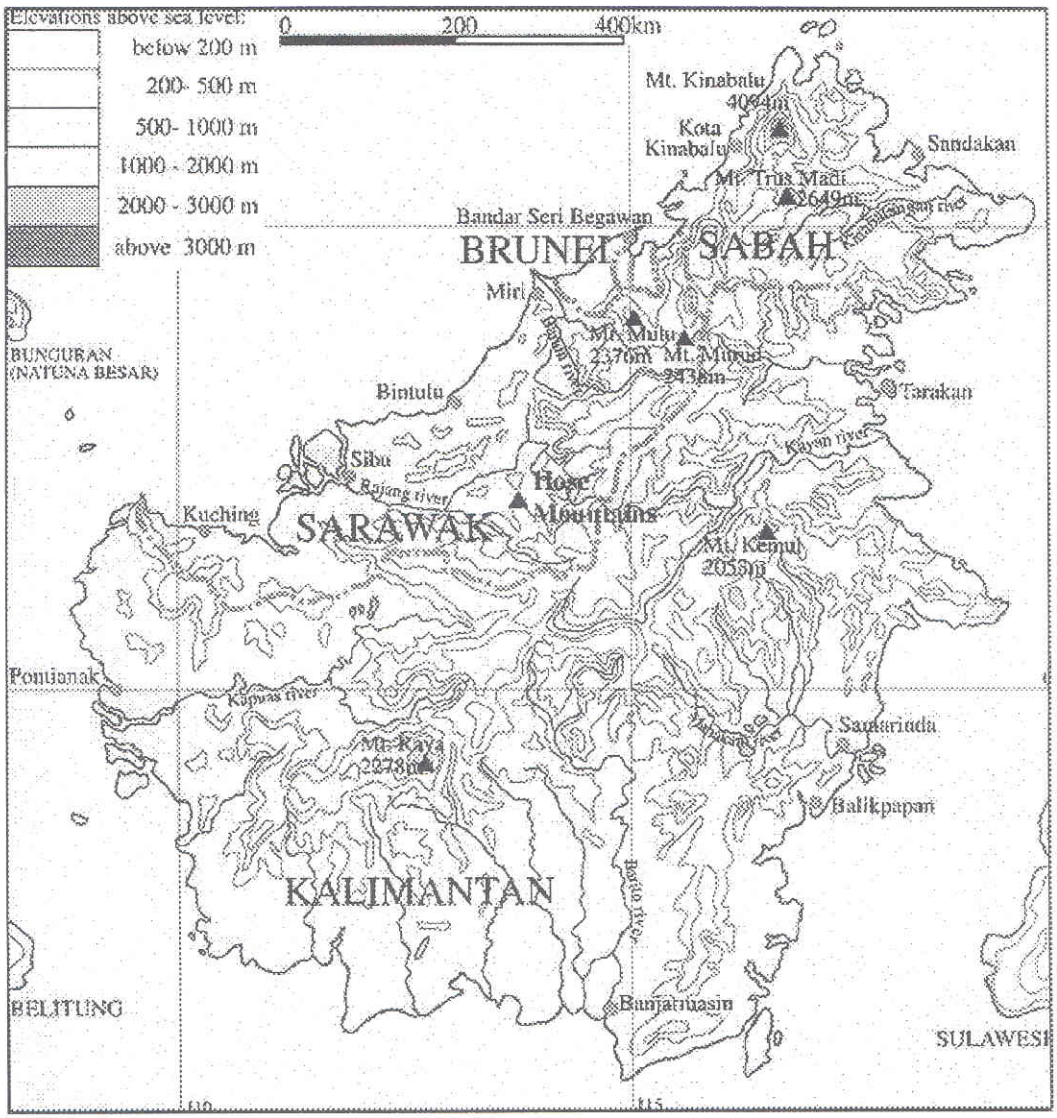

Figure1. Map of Borneo

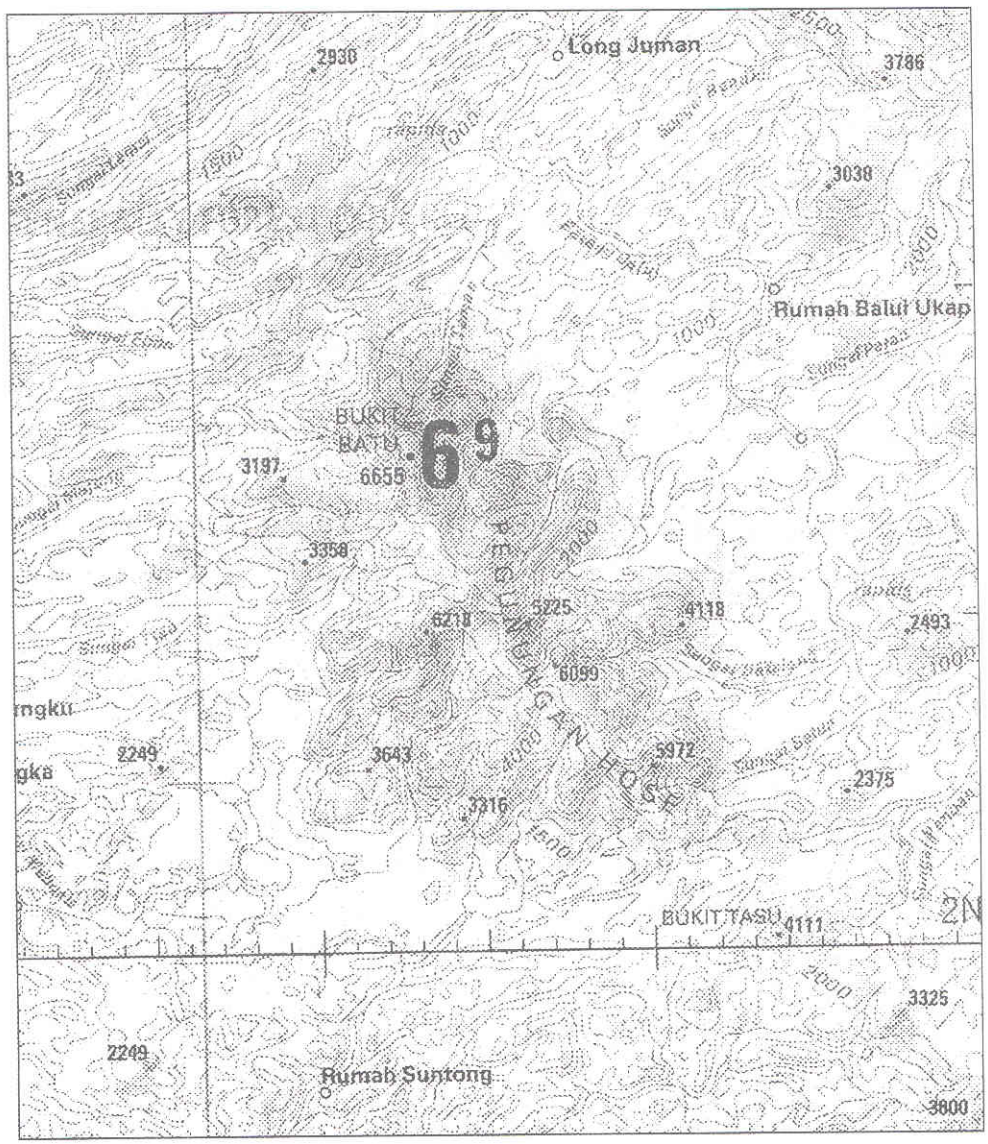

Figure 2. Map of Hose Mountains

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Chromosome studies in *Drosera* (Droseraceae)

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Abstract. Thirty-two species and one hybrid of *Drosera*, *Aldrovanda vesiculosa*, *Dionaea muscipula* and *Drosophyllum lusitanicum* were investigated for a chromosome analysis with sequentially fluorescent distamycin A and chromomycin A_3 (DMA-CMA), and actinomycin D and 4'-6-diamidino-2-phenylindole (AMD-DAPI) staining methods. In genus *Drosera*, no primary constriction was observed in the mitotic-metaphase chromosomes stained by orcein, DMA-CMA and AMD-DAPI. The hexaploid *D. spathulata* (2n=60) had 20 middle-size chromosomes stained positively by DMA-CMA and stained negatively by AMD-DAPI. Moreover, genomic *in situ* hybridization to mitotic chromosomes showed that 20 middle-size chromosomes of the hexaploid in *D. petiolaris* exhibited six bivalent and two unbivalent chromosomes at meiotic metaphase I. The bivalent chromosomes of *D. petiolaris* showed circular ring-shaped pairing and distinctively displayed four chromatids held together end-to-end association as a diffused-centromeric nature. In contrast, the bivalent chromosomes of *D. rotundifolia* showed ring-shaped pairing at end-to-end of two chromosomes as a behavior of the usual pairing of the localized-centromeric chromosome during meiosis I. Thus, respective, meiotic chromosomes of *D. rotundifolia* could perform a single active site out of multicentromeres or the diffused centromeres, although its mitotic chromosome has the active diffused-centromere throghout the length of the chromosome. Localization of active centromere in the meiotic chromosome might meke the species of *Drosera* in the Northern Hemisphere very stable and promoted a polyoloid speciation with the basic chromosome number of x=10.

Introduction

Drosera L. is a member of the Droseraceae and one of the largest genera among the carnivorous plants. *Drosera* is distributed throughout the world; more than 15 species are native to the Northern Hemisphere and more than 70 species are native to the Southern Hemisphere. It has the close relatives of the monotypic *Aldrovanda* L., *Dionaea* Ellis and *Drosophyllum* link. within the family (Diels 1906).

Most of the privious cytological investigation concentrated on a orcein karyomorphological study in *Drosera*. These studies of *Drosera* species were mostly of chromosome counts and karyotype analysis (*e.g.*, Kondo 1973, 1976, Kondo *et al.* 1976). The chromosome analysis in these studies disclosed coexistence of polyploid and aneuploid complex in genus *Drosera*, common nature of mitotic chromosomes with unclear primary constriction, and intraspecific high-similarity of each chromosome morphology. Although chromosome data in *Drosera* were so much accumulated, the conventional karyotype analysis was not possible to obtain more useful information from *Drosera* chromosomes since the chromosomes of this genus could not be distinguished using primary constriction. Thus, more critical chromosome analysis with banding technique and cytomolecular methods are necessary for further *Drosera* cytological investigation.

In this study, DNA base specific banding and *in situ* hybridization were employed for providing detailed data of karyomorphological characterization, and for clarifying

chromosomal phylogenetic relationships in the Droseraceae. Moreover, to demonstrate the diffused centromeric nature of the *Drosera* chromosomes, induction of fragment chromosomes by radiation was curried out.

Materials and Methods

Thirty-two species and one natural hybrid (*D. filiformis* x *D. intermedia*) of *Drosera* and *Aldrovanda vesiculosa*, *Dionaea muscipula*, *Drosophyllum lusitanicum* of the Droseraceae used in this study are listed in Table 1.

To observe mitotic chromosomes, root tips were collected and pretreated with 0.002 M hydroxyquinoline for two hours at 18°C before fixation with 45 % acetic acid for five minutes. Then, these were hydrolyzed in a mixture of 1N hydrochloric acid and 45% acetic acid (2:1) at 60°C for seven seconds. For, orcein staining, root meristems were cut and stained with 1 % aceto-orcein for six hours and then squashed. To observe meiotic chromosomes, pollen mother cells were stained and prepared with the standard aceto-orcein smear method. For slide preparation before fluorescent staining and *in situ* hybridization, root tips were pretreated, fixed and hydrolysed by the procedure described above, then they were squashed in 45 % acetic acid. The prepatations were air-dried for 24 hours at room temparature after removal of coverslips with dry ice. For Sequentially fluorescent staining with distamycin A and chromomycin A_3 (DMA-CMA) and with actinomycin D and 4'-6-diamidino-2-phenylindole (AMD-DAPI), the method of Schweizer (1981) and Schweizer (1976) was followed with slight modifications, respectively.

Hexadecyltrimethylammoniumbromide (CTAB)-based method was employed to isolate total genomic DNA of *Drosera rotundifolia*. Isolated DNA was treated with DNase-free RNase A (0.2 mg/ml) at 37 °C for one hour followed by extractions with phenol-chloroform and chloroform. Total genomic DNA was labeled with biotin-14-dATP by nick translation according to the supplier's instruction (BRL). Probe mixture (400 μl) contained 10 % dextran sulfate, 50 % formamide, 2xSSC (1xSSC; 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0) and 1 μg biotin-labeled DNA.

For *in situ* hybridization to chromosomes, biotin-labeled probes were cytochemically detected using the avidine-horse radish peroxidase complex (Vector Lab.) and a diaminobenzidene-H_2O_2 solution. The chromosomes were counterstained with 2 % Giemsa in 2xSSC for two minutes and were, then, mounted with Eukitt. All *Drosera* chromosomes at mitotic metaphase could not be classified by the method using localized-centromeric position, since they do not have localized centromere and/or primary constriction (Kondo *et al.* 1976, Kondo and Segawa 1988). Thus, individual chromosome areas stained by AMD-DAPI at mitotic metaphase were measured by an image analyzer (IBAS: Kontron). The karyotype formulae are based on measurements of 30 metaphase cells with DAPI single staining. Chromosome size are defined as: Super large (LL) > 15.00 μm^2 in area, large (L) 6.00-14.99 μm^2, middle (M) 1.50-5.99 μm^2 and small (S) <1.49 μm^2. Relative area of average-sized individual chromosome was calculated by average individual chromosome area/total area of the chromosoes in the complement x 100. The ratio of the largest chromosome to the smallest chromosome was calculated by average area of the largest chromosome / average area of the smallest chromosome.

Plants of *Drosera petiolaris* and *D. rotundifolia in vitro* were exposed to Gamma radiation in the Facility of Faculty of Engineering, Hiroshima University. Doses were determined by time and distance from the source around a circular disc in the 'Gamma shine' (Co unit). Soon after exposed to Gamma radiation, the flasks were returned to the culture room of the Laboratory for continuous *in vitro* culture.

Results and Discussion

Chromosomal analysis of 32 species and one natural hybrid of *Drosera*, *Aldrovanda vesiculosa*, *Dionaea muscipula* and *Drosophyllum lusitanicum* are shown in Table 1 and Fig. 1.

In the squash method skipped pretreatment, the sister chromatids of the *Drosera* species laid parallel to each other at mitotic-prophase to metaphase. The configuration of a chromatid complement at late anaphase was dome-shape or flat when viewed from the lateral side. This configuration indicated parallel separation at mitotic anaphase. The metaphase chromosome morphology and anaphase chromatid behavior supported that *Drosera* species have diffused-centromeric chromosome. Diffused-centromeric nature of the chromosome in *Drosera* was previously reported (Kondo *et al.* 1976, Kondo and Lavarack 1984). The presence of diffused-centromeric chromosomes in *Drosera* (Kondo *et al.* 1976, Kondo and Lavarack 1984, Kondo and Segawa 1988) is only a case in the dicots. In contrast, the sister chromatids of *Dionaea muscipula* laid parallel to each other at mitotic-prophase to metaphase and were not observed to move apart parallel in separation at mitotic anaphase since the chromatids showed the end-to-end association and their CMA-negative or DAPI-positive sites separated at last at mitotic latemetaphase. Thus, *Dionaea muscipula* could have a localized-centromere at the CMA-negative or DAPI-positive site of the chromosome. *Drosophyllum lusitanicum* had well-differentiated and localized centromeres in the chromosome at mitotic metaphase. *Aldrovanda vesiculosa* did not obviously show any localized centromere due to difficulty in observing non-staining gap of small chromosome in this genus.

In genus *Drosera*, the sixteen species and one hybrid in sect. Rossolis, ser. Eurossolis studied exhibited karyomorphologically the gradient type of the mitotic chromosome, while five species in section Rossolis, ser. Lasiocephala exhibited the interstitial type of the mitotic chromosome. These two series showed karyomorphologically different propaties each other. In chromosome number, the basic chromosome number of x=10 was directly connected to the species of ser. Eurossolis divided into three groups: The first group is characterized by the chromosome number of 2n=20, the second group is characterized by the chromosome number of 2n=40, and the third group is characterized by the chromosome number of 2n=80. In contrast, aneuploid chromosome numbers were characteristic in ser. Lasiocephala. Moreover, ser. Eurossolis has small and middle-size chromosomes, while ser. Lasiocephala has large-size chromosomes. From these cytogenetic points, ser. Eurossolis and ser. Lasiocephala took evolutionally indipendent differentiation each other, and then ser. Eurossolis might not be closely related to ser. Lasiocephala.

Average chromosome size made it possible to divide the members to sect. Rossolis, ser. Eurossolis into two major groups: One group, which was characterized by middle-size chromosomes, consisted of *D. anglica*, *D. brevifolia*, *D. capillaris*, *D. filiformis*, *D. intermedia*, *D. rotundifolia* and *D.* x *hybrida*, distributed in the Northern Hemisphere (Fig. 1). The other group, which was characterized by small-size chromosomes, consisted of *D. aliciae*, *D. capensis*, *D. dielsiana*, *D. hilaris*, *D. madagascariensis*, *D. montana*, the diploid and the tetraploid *D. spathulata*, *D. trinervia* and *D. villosa*, distributed mainly in the Southern Hemisphere (Fig. 1). Thus, correlation between average area of respective complement and distribution pattern seemed to be found in *Drosera*, sect. Rossolis, ser. Eurossolis.

The karyotype formulae and fluorescent staining in South African and South American species with the chromosome number of 2n=40 of *Drosera* suggested two possibilities of the basic chromosome number: One basic chromosome number might be x=10 because average area of respective chromosome complement of South African and South American species

were as small as those of the diploid *D. spathulata*. The other possible basic chromosome number might be x=20, because all species distributed in South Africa and South America studied displayed the chromosome number of 2n=40, exhibited up to two sat-chromosomes and did not have four sat-chromosomes in fluorescent staining. Moreover, the meiotic configulation of 20_{II} was observed in *D. madagascariensis* from South Africa. The phenomena of spontaneous hybridization and chromosome doubling for amphiploid speciation supported the species relationship with the basic chromosome number of x=10 (Kondo 1971, 1973). Thus, these results speculated that the species with chromosome number of 2n=40 in South Africa and South America might be arisen from diploid species with the basic chromosome number of x=10 by spontaneous hybridization to produce amphidiploid.

Among six populations of *Drosera spathulata* complex investigated, the chromosome numbers of 2n=20, 40 were counted from the Southern Hemisphere populations, while the chromosome numbers of 2n=40, 60 were counted from the Northern Hemisphere populations. Rattenbury (1957) observed the somatic chromosome number of 2n=20 in *D. spathulata* in a New Zealand population. Kondo (1971) observed a somatic chromosome number of 2n=40 in the same species in an Australian population. Migration trend of the *D. spathulata* complex was speculated by Kondo (1971) that the tetraploid *D. spathulata* seemed to be originated from the diploid *D. spathulata* in New Zealand and might be shown the northward distribution. Thus, the present result supported Kondo's explanation (1971).

Drosera petiolaris complex of sect. Rossolis, ser. Lasiocephala, displayed large total chromosome area more than 80.00 μm^2, and large average area of chromosome complement more than 7.00 μm^2 (Fig. 1). the *D. petiolaris* complex displayed both an aneuploid and polyploid nature since it showed chromosome numbers of 2n=12-14 in *D. petiolaris* and 2n=24 in *D. ordensis*. Kondo (1976) and Kondo and Lavarack (1984) reported the chromosome number of 2n=12 in *D. dilatato-petiolaris*, *D. falconeri* and *D. petiolaris* and 2n=19 [hypertriploid as Kondo (1984) said] in *D. lanata*. Kondo (1976) suggested that primitive basic chromosome number of x=6 for *Drosera* was *D. petiolaris*. Moreover, Sheikh and Kondo (1995) reported that species of sect. Lamprolepis possessed large-sized chromosomes with aneuploid series. Thus, cytological studies suggest that *D. petiolaris* complex and species of sect. Lamprolepis had common karyomorphological features with low basic chromosome number.

Drosera x *hybrida* is a natural hybrid between *D. filiformis* and *D. intermedia*. In florescent staining investigation at metaphase, *D. filiformis* had four chromosomes with CMA faintly-positive or DAPI-positive distal bands and 16 chromosomes with CMA faintly-positive or DAPI-positive interstitial bands, while *D.* x *hybrida* had two chromosome with CMA faintly-positive or DAPI-positive distal bands, eight CMA-faintly positive or DAPI-positive interstitial bands, and ten chromosomes with neither CMA nor DAPI band. Moreover, *D. intermedia* displayed no CMA-negative or DAPI-positive band in all chromosomes. Thus, DNA fluorescent banding technique had an ability to distinguish between two parental chromosomes in *D.* x *hybrida*.

Total genomic DNA from *Drosera rotundifolia* hybridized 20 middle-size chromosomes of the hexaploid *D. spathulata* (2n=60) by genomic *in situ* hybridization (GISH). Thus, the hexaploid *D. spathulata* could be arisen from an amphidiploid hybrid origin between the sympatric species of the diploid *D. rotundifolia* (2n=20) and the tetraploid *D. spathulata* (2n=40). Moreover, the hexaploid *D. spathulata* had 20 CMA-positive or DAPI-positive middle-size chromosomes and 40 CMA faintly-positive or DAPI-positive small-size chromosomes. The tetraploid and the hexaploid *D. spathulata* and the diploid *D. rotundifolia* are sympatric in some areas in Shizuoka Prefecture in Japan (Kondo 1971).

The hexaploid *D. spathulata* 'Kansai type' has been hypothesized to be derived from a hybrid between *D. rotundifolia* and the tetraploid *D. spathulata*, since it is morphologically intermediate and shows a bimodal karyotype (Kondo 1971, Kondo and Segawa 1988). The GISH with total DNA of *D. rotundifolia* supported the Kondo's hypothesis of hybrid-origin of the hexaploid *D. spathulata* 'Kansai type' (Kondo 1971, Kondo and Segawa 1988). Further molecular phylogenic investigation is expected to identify a parent involved in a maternal or cytoplasmic inheritance of the hexaploid *D. spathulata*.

Drosera petiolaris and *D. rotundifolia* both *in vitro* exposed to different doses of Gamma radiation showed fragmented and fused chromosomes. Different types of the chromosome fragmentation could be observed in root tip cells of the exposed plants. Simple breakages occurred frequently in most of the metaphase cells exposed to 50 Gy produce chromosome fragments with various length. Long chromosomes made by fusion of two or more chromosomes were also found in some metaphase cells radiated. The plant materials *in vitro* exposed to above 1000 Gy were completly died 120 days after the exposure. Separation of the fragment chromosomes at mitotic anaphase and telophase was quite nomal. The localized-centromeric chromosomes always displayed lagging chromosomes and/or chromosome bridges at mitotic anaphase and micronuclei at mitotic telophase after irradiation. Most of the fragment chromosomes in the somatic cells of *Drosera* did not lose kinetic activity and thus, might have multi- or diffused-centromeres, since the behaviour and appearence of the chromosomes are quite in agreement with some of the featuers of holocentric and/or diffused-centromeric chromosomes reported previously (Tanaka and Tanaka 1977, Sheikh *et al.* 1995).

The 2n=14 plant from the intraspecific aneuploid series in *D. petiolaris* (2n=12-14) exhibited six bivalent and two unbivalent chromosomes at meiotic metaphase I. In contrast, *D. rotundifolia* (2n=20) in a polyploid series with the basic chromosome number of x=10 in the Northern Hemisphere exhibited ten bivalent chromosomes at metaphase I of meiosis. The bivalent chromosomes of *D. petiolaris* showed circular ring-shaped pairing and distinctively displayed four chromatids held together end-to-end association. In contrast, the bivalent chromosomes of *D. rotundifolia* showed ring-shaped pairing at end-to-end of two chromosomes as a behavior of the usual pairing of the localized-centromeric chromosome during meiosis I. Thus, respective, meiotic chromosomes of *D. rotundifolia* could perform a single active site out of multicentromeres or the diffused centromeres, although its mitotic chromosome has the active diffused-centromere throghout the length of the chromosome. Localization of active centromere in the meiotic chromosome might make the species of *Drosera* very stable and promoted a polyoloid speciation with the basic chromosome number of x=10. Bivalent chromosomes at meiotic metaphase I in some species of *Luzula* (Malheiros and Castro 1947, Nordenskiöld 1951) and *Elocharis* (Strandhede 1965) were equatorially oriented with regared to the spindle at meiotic metaphase I and separate equationally at anaphase I. This type of orientation of the holocentric chromosomes at meiotic metaphase I (White 1973). Speciation accompanied by orderly and stable polyploidization in *Drosera* seen in the Northern Hemisphere could be an unexpected phenomenon and could be due to localized-centromeric behavior function and formation during the meiosis I insted of diffused-centromeric nature seen during the mitosis. The species of *Drosera* in the Northern Hemisphere might obtain a character of the most active centromere among the multi- or poly-centromeres or along the entire lengths of the chromosomes to maintain the stable meiotic division for the stable sexual reproduction after the ancestor species might choose rarely the basic chromosome number of x=10 under certain natural selective pressure.

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Table 1. Comparison in karyotypes of 32 species and one natural hybrid of *Drosera*, *Aldrovanda vesiculosa*, *Dionaea muscipula* and *Drosophyllum lusitanicum* investigated

Species	Chromosome number	Total chromosome area (μm^2) (mean ± SD)	Chromosome area from the largest to the smallest chromosomes (μm^2)	Average area of chromosome complement (μm^2) (mean±SD)	Largest chromosome / smallest chromosome	inter-chromosomal asymmetry index	Karyotype formula[a]	Number of CMA- positive or DAPI-negative sat-chromosomes in metaphase complements
Genus *Drosera*								
Subgenus Rorella								
Section Thelocalyx								
D. burmanni	20	15.36 ± 3.12	1.02 - 0.57	0.77 ± 0.12	1.84	0.16	20S	2
D. sessilifolia	80	48.15 ± 6.94	0.92 - 0.35	0.62 ± 0.14	2.67	0.24	80S	2
Section Arachnopus								
D. adelae	30	16.59 ± 2.92	0.95 - 0.22	0.55 ± 0.17	4.87	0.32	30S	2
D. indica	28	47.18 ± 9.04	2.36 - 0.45	1.69 ± 0.44	5.97	0.26	22M + 6S	4
D. prolifera	30	19.94 ± 5.02	1.04 - 0.24	0.66 ± 0.18	4.72	0.27	30S	2
Section Rossolis								
Series Eurossolis								
D. aliciae	80	75.96 ± 10.26	1.49 - 0.41	0.95 ± 0.23	4.02	0.25	80S	2
D. anglica	40	93.59 ± 29.56	3.19 - 1.71	2.34± 0.34	1.95	0.15	40M	0
D. brevifolia	20	38.15 ± 5.09	2.48 - 1.52	1.91 ± 0.25	1.65	0.14	20M	2
D. capensis	40	21.50 ± 5.73	0.82 - 0.34	0.53 ± 0.10	2.56	0.20	40S	2
D. capillaris	20	68.50 ± 12.90	4.80 - 2.33	3.43 ± 0.66	2.08	0.20	20M	0
D. collinsiae	40	38.11 ± 7.01	1.06 - 0.43	0.70 ± 0.14	2.52	0.21	40S	0
D. dielsiana	40	37.52 ± 10.10	1.35 - 0.59	0.94 ± 0.19	2.38	0.21	40S	4
D. filiformis	20	88.03 ± 19.07	5.87 - 2.97	4.40 ± 0.82	2.01	0.19	20M	2
D. hilaris	40	28.91 ± 8.95	1.13-0.39	0.72 ± 0.16	3.04	0.23	40S	2
D. intermedia	20	47.33 ± 13.82	3.26-1.63	2.37 ± 0.44	2.02	0.19	20M	2
D. madagascariensis	40	20.60 ± 5.72	0.80 -0.29	0.51 ± 0.12	2.93	0.25	40S	2
D. montana	40	50.01± 11.99	1.95 -0.59	1.25 ± 0.34	3.37	0.27	10M + 30S	2
D. rotundifolia	20	46.28± 9.19	3.18-1.52	2.31 ± 0.42	2.19	0.19	20M	0
D. spathulata	20	17.79± 4.79	1.25- 0.59	0.89 ± 0.16	2.17	0.19	20S	2
	40	36.74± 6.71	1.40- 0.52	0.92 ± 0.21	2.78	0.23	40S	0
	60	92.49±13.68	3.73- 0.52	1.54 ± 1.05	7.64	0.68	20M + 40S	0
D. trinervia	40	35.39 ± 8.71	1.90- 0.48	0.88 ± 0.27	4.60	0.32	2M + 38S	2
D. villosa	40	27.99± 6.14	1.14- 0.33	0.70 ± 0.19	3.60	0.27	40S	2
D. x hybrida	20	62.88 ± 8.59	5.50 -1.50	3.14 ± 1.27	3.70	0.41	20M	2
Series Lasiocephala								
D. dilatato-petiolaris	12	98.07 ± 18.65	9.77- 6.24	8.17 ± 0.98	1.57	0.12	12L	0
D. falconeri	12	89.12 ± 19.33	8.87- 6.34	7.43 ± 0.70	1.40	0.10	12L	0
	13	95.12 ±12.34	9.13- 3.02	7.32 ± 1.52	3.22	0.21	12L + 1M	0
D. lanata	12	89.63 ±15.62	8.94 - 6.10	7.47 ± 0.83	1.48	0.12	12L	0
	14	100. 80 ± 14.88	9.15- 3.05	7.20 ± 1.81	3.06	0.25	12L + 2M	0
D. ordensis	24	181.79 ±35.24	9.86 - 6.06	7.57 ± 1.04	1.64	0.14	24L	0
D. petiolaris	12	90.52 ±19.42	9.01 - 6.00	7.54 ± 0.89	1.52	0.12	12L	0
	13	93.34 ± 17.62	9.04 - 2.96	7.18 ± 1.45	3.22	0.21	12L + 1M	0
	14	102.66 ±15.08	9.34 - 3.09	7.33 ± 1.88	3.14	0.26	12L + 2M	0
Section Stelogyne								
D. hamiltonii	28	18.88 ± 5.19	1.00 - 0.42	0.67 ± 0.14	2.42	0.21	28S	2
Section								
D. binata	32	41.06 ±10.02	1.89 - 0.75	1.28 ± 0.28	2.67	0.22	8M + 24S	2
Subgenus Ptycnostigma								
Section Ptycnostigma								
D. cistiflora	60	34.10± 6.63	0.83 - 0.28	0.57 ± 0.11	5.63	0.21	60S	4
D. pauciflora	40	23.42± 5.33	1.76 - 0.21	0.59 ± 0.39	9.62	0.70	2M + 38S	4
Subgenus Ergaleium								
Section Polypeltes								
D. auriculata	32	30.24 ± 5.56	1.49 - 0.48	0.95 ± 0.26	4.28	0.30	32S	0
D. peltata	32	28.03 ± 9.68	1.40 - 0.39	0.88 ± 0.27	4.39	0.32	32S	4
Genus *Aldrovanda*								
Aldrovanda vesiculosa	48	43.25 ± 7.50	1.48 - 0.33	0.90 ± 0.26	6.37	0.30	48S	2
Genus *Dionaea*								
Dionaea muscipula	33	96.96± 19.03	3.82 - 0.53	2.94 ± 0.53	9.20	0.20	32M + 1S	0
Genus *Drosophyllum*								
Drosophyllum lusitanicum	12	377.28± 29.15	40.02 -26.28	31.44± 5.20	1.32	0.18	12LL	0

[a] S = small-size chromosome (<1.49μm^2), M = middle-size chromosome (1.50 - 5.99 μm^2), L = large-size chromosome (6.00 - 14.99 μm^2), LL = super large-size chromosome (> 15.00 μm^2)
SD = standard deviation of the mean

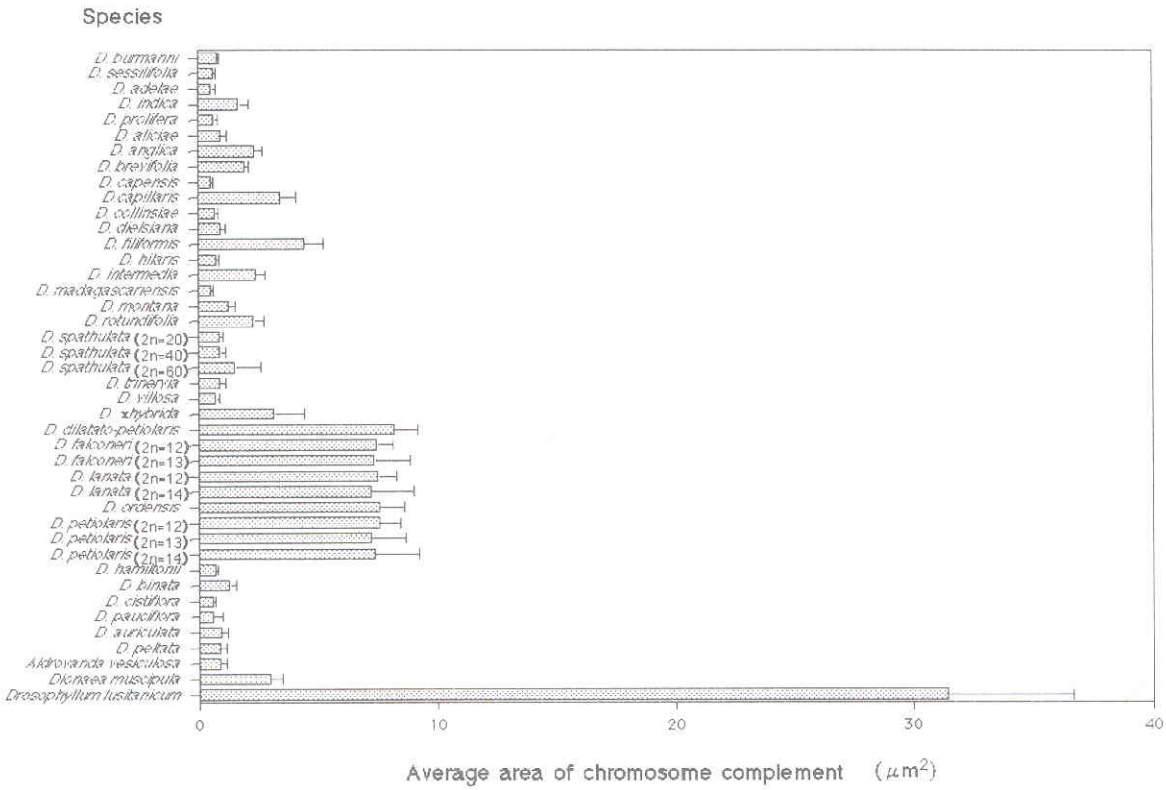

Figure 1. Histograms of average chromosome size measured in the members of the Droseraceae studied. Average chromosome size made it possible to divide the members to sect. Rossolis, ser. Eurossolis into two major groups: One group, which was characterized by middle-size chromosomes, consisted of *D. anglica*, *D. brevifolia*, *D. capillaris*, *D. filiformis*, *D. intermedia*, *D. rotundifolia* and *D.* x *hybrida*, distributed in the Northern Hemisphere. The other group, which was characterized by small-size chromosomes, consisted of *D. aliciae*, *D. capensis*, *D. dielsiana*, *D. hilaris*, *D. madagascariensis*, *D. montana*, the diploid and the tetraploid *D. trinervia* and *D. villosa*, distributed mainly in the Southern Hemisphere. In the species of *Drosera* studied, *D. petiolaris* complex had more than 7.00 μm^2 of large-size chromosomes.

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Observations on prey preference and other associations of *Aldrovanda vesiculosa* in a new culture system

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Abstract. *Aldrovanda vesiculosa*, also known as the waterwheel plant, is an aquatic free-floating carnivorous plant noted for its difficulty of cultivation. A new method of cultivation allows for the rapid propagation of *A. vesiculosa* without using complex additives, and this method is effective with plants from Europe, Asia, and Australia. This involves the creation of a complex periphyton and associated community, including invertebrate animals and protists over a rich soil substrate. To better understand the associations of *A. vesiculosa* with protists and invertebrate animals in this system, two lines of investigation were pursued. The first line of investigation involved examination of whether *A. vesiculosa* is selective of the prey that it traps, and if so, what characteristics the different types of trapped prey share. Several prey items were offered to the plants, and observations were made for 1 wk with amphipods and snails preyed upon the most, perhaps due to their seeking shelter in or on the plants of *A. vesiculosa*. The second line of investigation involved the examination of microscopic animals and protists living with *A. vesiculosa* in this new system of cultivation to determine if the types of organisms associated with the periphyton may indicate any salient features of the new system. Large numbers of rotifers, testate amoebae, and other organisms indicative of mesoeutrophic or eutrophic conditions were observed.

Introduction

Aldrovanda vesiculosa (Droseraceae), also known as the waterwheel plant, is an aquatic free-floating carnivorous plant found growing just below the surface of the water. It is native to Australia, Europe, Africa, and Asia, first being discovered in 1699 by Leonard Plunkenet. Europe's population of *Aldrovanda* has become endangered in recent years due to pollution, and in Japan *Aldrovanda* has gone extinct in the wild (D'Amato 1998).

A simple method of cultivating *A. vesiculosa* has recently been described (Darnowski 2002) which uses rich garden soil and companion plants with abundant roots to create an environment in which *A. vesiculosa* plants from Europe, Asia, and Australia rapidly grow and reproduce. To better understand this method, which includes the formation of a periphyton mat with associated organisms, the nature of the prey trapped by *A. vesiculosa* and the nature of the animals and protists associated with *it* may provide significant clues. That a periphyton mat is produced may not be surprising given the observation that in the wild in the Northern Territory, Australia, *A. vesiculosa* can be seen growing among large floculant particles (Darnowski, unpublished observation), which may represent a periphyton mat along with other organisms.

Waterwheel plants can reach a maximum length of 20 cm and have whorls of leaves perpendicular to its stem. These leaves bear traps that prey on small aquatic organisms such as *Daphnia* and amphipods. Each whorl usually consists of eight leaves with a trap at each leaf's apex. As the plant grows and produces more whorls, the aged whorls at the basal end soften and fall from the plant. Under favorable conditions this plant is able to grow at a rate of 0.4 to 0.9 cm d^{-1} (Breckpot 1997).

Most carnivorous plants, including, *A. vesiculosa*, live in nutrient poor environments, which is thought to be the reason for their carnivory. *A. vesiculosa* inhabits shallow bodies of water such as lakes, bogs, lagoons, and billabongs that are dystrophic with sparse bottom fauna. Filamentous algae and competition from denser stands of plants can lead to the disappearance of

this plant in a habitat. Other carnivorous plants that share the same habitat as *A. vesiculosa* include various bladderworts (Lowrie 1998; Adamec 1995).

The trapping mechanism of *A. vesiculosa* is nearly unique among carnivorous plants since only one other plant, the Venus Fly Trap (*Dionaea muscipula*), utilizes this same technique known as the spring or beartrap (Gentle 1996). The spring trap's shape is similar to that of a bivalve, with two semicircular halves usually measuring 4 mm long and 2.5 mm wide. Possible prey items such as *Daphnia* or amphipods are attracted to the plant by four to eight long apical bristles on which they can seek shelter. Although these are important in bringing organisms into contact with the plant, the primary purpose of these bristles is to prevent the traps from capturing floating debris near the surface of the water (Breckpot 1997). The trap is stimulated when an organism touches one of the forty small trigger hairs that the trap possesses on its inner margins (Lowrie 1998). The trap shuts in only 20 msec as a result of an action potential traveling across the excitatory cells in one of the most rapid movements seen in plants (Iijima and Sibaoka 1981).

After the leaf ingests the prey, approximately sixty to eighty tiny teeth inside the trap lock together and excess water is pumped out. Last, the plant releases a secretion to seal the trap shut. Digestion takes place over a few days as the digestive glands on the inside walls of the trap secrete enzymes and acid to dissolve the organism. After the organism is dissolved, its nutrients are absorbed. Predation is not affected by the digestive process, allowing traps to catch more organisms even if there is already prey in a trap (D'Amato 1998). *A. vesiculosa* will consume anything that touches the trigger hairs stimulating the trap to shut even if the organism is larger than the trap. When this scenario occurs, the trap will remain closed and eventually die (Gentle 1996).

Much research has been conducted on *A. vesiculosa*, but one area of study that has not been given much attention is that of prey selection. It is known that the plant eats different organisms but in what proportions is not known. In order to determine if the plant does select which prey it captures when different species of aquatic organisms are available, an experiment was carried out using several prey items that were offered to the plant in a laboratory setting.

Similar studies have been performed on other aquatic carnivorous plants from the genus *Utricularia*. Like *Aldrovanda*, *Utricularia* sits and waits for prey to approach it. Hence, it has been proposed that prey selection probably depends more on the characteristics of the prey, like its activity and swimming patterns, and not on the behavior of the predator (Harms and Johansson 2000). In three sympatric species of *Utricularia*, planktonic prey were preyed upon in smaller amounts than what was available while prey living on a substrate, plant or sediment, were captured in proportion to availability (Harms 1999).

Others have observed that prey selection and carnivory may not be important aspects in one species, *U. purpurea*. A substantial amount of biomass is invested to develop and maintain traps but they only capture a small number of living organisms. Rather than a predator-prey relationship, a mutualistic relationship was observed between mature bladders and algae and zooplankton (Richards 2001). There may be similar connections between occupants of the periphyton and *A. vesiculosa* in culture.

Materials and Methods

Cultivation of *Aldrovanda* *A. vesiculosa* was cultivated in the laboratory to make a stock supply according to Darnowski (2002). 8 l aquaria were used, filled with approximately 5 cm inches of garden soil from Queen Anne's County, Maryland, USA. This soil, clay-based with organic amendments, had added to it double distilled water to a depth of approximately10 cm. One or two water hyacinths (*Eichhornia crassipes*; Pontideriaceae) were added to each tank, and several segments of *A. vesiculosa* were placed on the surface of the water. The large root mass of these

plants may help to condition the water for *A. vesiculosa*. The condition of the plants and tanks were observed periodically and more distilled water was added when necessary to maintain the water level.

Selection and description of prey items Based on their size relative to that of the traps, prey items were selected. *Daphnia magna*, copepods, and amphipods were ordered from Ward's Natural Science (Chicago, Illinois, USA) and mosquito larvae, baby pond snails (*Amnicola limnosa*) , and rotifers were purchased from Carolina Biological Supply Company (North Carolina, USA). Some pond snails, already present in tanks used to culture *A. vesiculosa* before experiments were performed, were also included among prey organisms.

These prey can be divided into three size classes, the smallest being the rotifers which were microscopic and which have been found to live in the traps of *U. purpurea* (Richards 2001). The copepods and *Daphnia magna* were slightly larger, approximately 0.5 -1.5 mm long. The largest class of prey ranging from 2-3 mm included the amphipods, baby snails, and mosquito larvae. Prey organisms were fed a 1:1 mix of Brewer's yeast and Spirulina (Debittered Brewer's Yeast, Now Foods, Bloomingdale, IL USA; Spirulina, Earthwise Nutritionals, Petaluma, CA, USA) until used in experiments.

Daphnia magna or water fleas are small crustaceans measuring 0.2-6 mm. These are found mainly in freshwater (Fox 1994). Copepods and amphipods are larger aquatic crustaceans measuring 1-2 mm and 2-50 mm, respectively (www.nmnh.si.edu/iz/copepod / and http://web.odu.edu/sci/biology/jrh/whatis.htm). The larval stage of mosquitoes is the second stage of their metamorphosis, lasting 1-3 wk. During this period they feed on bacteria and algae and brush-like hairs help them swim. Rotifers are microscopic organisms which use beating cilia to swim in ponds and lakes (Carolina Biological Supply Company 1998).

Experiments on selection of prey From the stock supply, robust pieces of *A. vesiculosa* containing six whorls were cut. Three pieces of the plant were used for each experiment. All three pieces were examined with a dissecting microscope prior to the experiment to record any organisms already present in the traps--any such organisms were subtracted from the totals in Tables 1-4. The plants were then placed in a small GladWare® container three quarters full of filtered pond water. The pond water used in these experiments was taken from containers in Washington College's greenhouse that had aquatic plants growing in them. The original water in these pots had aged over time, meaning minerals and nutrients had been taken up by the plants and small animals, resulting in water properties that would support *Aldrovanda* in the wild. A coffee filter was used to trap vegetation and organisms present in the pond water so that only conditioned pond water was used for experiments and so that no additional organisms from the greenhouse were included.

Prey were then added using a disposable plastic pipette. The first experiment involved amphipods, *Daphnia magna*, and copepods with five of each added per container. In the second experiment two additional prey items were used, mosquito larvae and rotifers. The same quantities of prey employed in the first experiment were also used here, except for mosquito larvae of which there was a limited supply, and the rotifers because they were much smaller than the other prey. Three mosquito larvae were added to *Aldrovanda* numbers 4 and 5 and only 2 to *Aldrovanda* number 6. 0.5 mL of rotifers (3.6×10^8 rotifers l^{-1}) were added to these three containers.

After determining which prey were caught in the greatest quantity, a third experiment was conducted to see if the predation pattern would change given the addition of other organisms of similar size and behavior. This last experiment involved only amphipods and baby snails. These two animals are similar in their behavior because they both periodically seek shelter in floating aquatic plants such as *A. vesiculosa*. The baby snails cling to the sides of a container or to plants in the water, and the amphipods actively seek plants to hide from predators. *Aldrovanda* plants 7-9 (Table 3) received two amphipods and two snails while *Aldrovanda* plants10-12 (Table

4) received the same number of snails but three times the number of amphipods. All containers in these experiments were then covered with plastic wrap to prevent any additional prey from entering the containers. The traps were then observed every 2 d under the dissecting microscope for 1 wk. Notes were made of what was in the traps and the condition of the plants.

Observation of associated animals and protists 20 l tanks, prepared according to Darnowski (2002), were prepared and placed in a frost-free greenhouse in Queen Anne's County, Maryland, USA, and allowed to grow for approximately 5-8 months. At the start, these tanks contained tap water, *A. vesiculosa* from a variety of locations (Europe, Girraween Lagoon in Australia's Northern Territory, Japan, Australia's New South Wales, southern Western Australia), water hyacinths, and bladderworts (*U. gibba* and other species). In addition, small volumes of water from active tanks of *A. vesiculosa* including both protists and invertebrate animals were added to promote the formation of the periphyton mat. This addition always included a red-orange cyanobacterial species.

During the late winter and early spring of 2002, samples of water and the periphyton mat were removed from these tanks, but especially the tank containing *A. vesiculosa* from Girraween Lagoon, Northern Territory, Australia and examined from 40-400x total maginfication using bright field microscopy. Images of the organisms present were taken using a 1.5 megapixel RCA digital camera pointed into one eyepiece of the stereo compound microscope. Organsisms were identifed using a variety of guides to protozoa and other organisms commonly associated with them (Patterson 1996).

Results

In the first experiment, only a small number of prey were caught in the traps. *A. vesiculosa* captured only *Daphnia* and amphipods, and mostly examples of the latter organism (Table 1). The second experiment yielded similar results to the first, only amphipods and *Daphnia* were caught (Table 2). All the plants caught prey with *A. vesiculosa* number 6 capturing the most, a total of three amphipods between the third and fourth whorls. Again, amphipods were the most commonly trapped prey. Several plants showed outgrowth of new shoots, a normal response to decapitation of the plants as was performed in preparing the experiments.

The least amount of prey caught was observed in the last experiment. *A. vesiculosa* numbers 8-11 did not catch any prey. *A. vesiculosa* number 7 caught an amphipod and a baby snail in the fourth whorl (Table 3), and *A. vesiculosa* number 12 caught one amphipod in the sixth whorl on the second day as illustrated in Table 4.

Associated animals and protists Many microscopic organisms were found in abundance in the cultures of *A. vesiculosa*. This being a qualitative study rather than a quantitative, the organisms occurring the most obvious numbers were recorded. Most obvious were testate, or shelled, amoebae, rotifers, and cyanobacteria.

Discussion

Selection of prey Based on the data, two conclusions can be drawn: 1) amphipods were preyed upon the most and 2) a majority of the traps did not catch any prey. A possible explanation as to why the amphipods were chosen over the other prey is their swimming behavior which was observed under the dissecting microscope. Frequently, the amphipods would seek shelter in the plant, thus increasing the probability of coming into contact with the traps and being caught. This pattern has previously been observed by Harms (1999) in the genus *Utricularia*, and it may explain the capture of a snail in the third experiment since the snails also come into close contact with the plants. Harms concluded that planktonic prey such as *Ceriodaphnia* and *Bosminia* were at a lower risk of being captured than prey living on the plant like copepods or ostracods. Why more

copepods were not caught is unclear and deserves further study, especially since these organisms are abundant in non-experimental cultures of *A. vesiculosa.*

Prey size in relation to trap size is another possible reason the amphipods were caught in greater quantities. Several of the *Daphnia magna* and mosquito larvae appeared to be larger than the traps so they were less likely to be caught, while there was a possibility that the rotifers were too small to even trigger the traps, also indicated by their ability to live in the traps of *U. purpurea* (Richards 2001). Careful observation for leaf absence, resulting from trap death after a prey item larger than the trap was caught, was made so this predation did not go unnoticed. However, this was not observed. Therefore, the size of the trap may determine the nature of the prey caught (Harms 1999).

Many of the traps did not catch any prey, perhaps because there was a limited amount of prey available for experiments. In the first experiment there were only 15 prey items compared to approximately 48 traps. The second experiment had 2 or 3 more prey items depending on the container plus the 0.5 mL of rotifers, yet similar results compared to those from the first experiment were seen. Prey was present in even smaller quantities for the third experiment and this was illustrated in the data.

Compared to the second experiment, the dramatic decrease observed in this experiment might have been because of soil clogging the traps and covering the trigger hairs, or the low availability and condition of the prey which was not optimal--this last experiment occurred later than the others, at a time when many prey cultures were no longer in excellent condition.

The effect of an already low abundance of prey could be enhanced if the prey were to die once in the container. Some prey might die naturally or perhaps because of attack by other prey species used in the same experiment. This activity was observed on one occasion between an amphipod and *Daphnia*, where the *Daphnia* was being eaten by the amphipod. If this process occured regularly than it would greatly decrease the probability of *Daphnia* being caught because a proportion of them would have been killed by the amphipods.

Another condition which might have affected the number of prey caught was the number of traps per whorl. Throughout the course of the experiment, 1 wk, several traps fell off each of the plants. This could be a result of a natural process as the plant grows or shock from being cut and placed in a new medium. Also, the number of leaves per whorl is dependent on how fast the plant develops. Younger plants might have less than eight leaves per whorl and these leaves are much smaller than more mature plants. In future, use of intact plants instead of sets of six whorls is indicated.

The last problem encountered that might have affected the data is difficulty in determining the contents of some traps. Several of the traps, especially in the third experiment, contained soil which made it difficult to see through them and determine if they were occupied prior to the experiment. A temporary drop in the water level brought the plants into close proximity with the soil bottom where they were able to catch soil particles. During the experiment most of this soil was released by traps as they opened to catch prey. In addition, several prey items were very small, such as the rotifers, making it difficult to determine what the trap had caught. These traps could have been dissected to identify the prey, but the plant would have been sacrificed, ruining the experiment.

In summary, this part of the study shows that *A. vesiculosa* does select prey based on characteristics of the prey. Prey behavior leading to increased contact with the plant, either living on the plant or seeking its shelter periodically, will increase the probability of predation for that species. The prey also has to be similar in size to the traps to stimulate the trigger hairs within the lobes.

Associated protists and Invertebrates

The protists and invertebrate animals which were found in successful cultures of *A. vesiculosa* from a variety of sites around the world indicate mesoeutrophic to eutrophic conditions, especially

the abundance of cyanobacteria and testate amoebae. This shows the need for precise chemical tests to determine the concentrations of various mineral nutrients in solution in these cultures, especially N, P, K, and B, in the study of this new culture method. In particular the question is raised of the role that these companion species may play in the ecology of *A. vesiculosa*, perhaps related to the non-carnivorous associations proposed for *U. purpurea* (Richards 2001).

General conclusion

The new method for cultivating *A. vesiculosa* (Darnowski 2002) shows great promise not only for producing many plants in a simple manner but also for studying important ecological associations of these plants, both associations with prey and associations with other organisms.

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Table 1. Number and type of prey caught in each whorl in the first experiment.

	Aldrovanda 1				Aldrovanda 2				Aldrovanda 3			
Whorl	Day 2	Day 4	Day 6	Day 7	Day 2	Day 4	Day 6	Day 7	Day 2	Day 4	Day 6	Day 7
1	0	0	0	0	0	Daphnia	0	0	0	0	0	0
2	0	0	0	0	0	0	0	0	0	0	0	0
3	0	0	0	0	0	0	0	0	0	0	0	0
4	0	0	0	0	0	0	0	0	0	0	0	0
5	0	0	0	0	0	0	0	0	0	0	0	0
6	0	0	0	0	0	Amphipod	0	0	0	Amphipod	0	Amphipod

Table 2. Number and type of prey caught in each whorl in the second experiment.

	Aldrovanda 4				Aldrovanda 5				Aldrovanda 6			
Whorl	Day 2	Day 4	Day 6	Day 7	Day 2	Day 4	Day 6	Day 7	Day 2	Day 4	Day 6	Day 7
1	0	0	0	0	0	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0	0	0	0	0	0
3	Amphipod	0	0	0	0	0	0	0	Amphipod (2)	Amphipod	0	0
4	Daphnia	0	0	0	0	Amphipod (2)	0	0	0	Amphipod	0	0
5	0	0	0	0	0	0	0	0	0	0	0	0
6	0	0	0	0	0	0	0	0	0	0	0	0

Table 3. Number and type of prey caught in each whorl of Aldrovanda plants 7-9 in the third experiment.

	Aldrovanda 7				Aldrovanda 8				Aldrovanda 9			
Whorl	Day 2	Day 4	Day 6	Day 7	Day 2	Day 4	Day 6	Day 7	Day 2	Day 4	Day 6	Day 7
1	0	0	0	0	0	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0	0	0	0	0	0
3	0	0	0	0	0	0	0	0	0	0	0	0
4	Amphipod	Snail	0	0	0	0	0	0	0	0	0	0
5	0	0	0	0	0	0	0	0	0	0	0	0
6	0	0	0	0	0	0	0	0	0	0	0	0

Table 4. Number and type of prey caught in each whorl of Aldrovanda plants 10-12 in the third experiment.

	Aldrovanda 10				Aldrovanda 11				Aldrovanda 12			
Whorl	Day 2	Day 4	Day 6	Day 7	Day 2	Day 4	Day 6	Day 7	Day 2	Day 4	Day 6	Day 7
1	0	0	0	0	0	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0	0	0	0	0	0
3	0	0	0	0	0	0	0	0	0	0	0	0
4	0	0	0	0	0	0	0	0	0	0	0	0
5	0	0	0	0	0	0	0	0	0	0	0	0
6	0	0	0	0	0	0	0	0	Amphipod	0	0	0

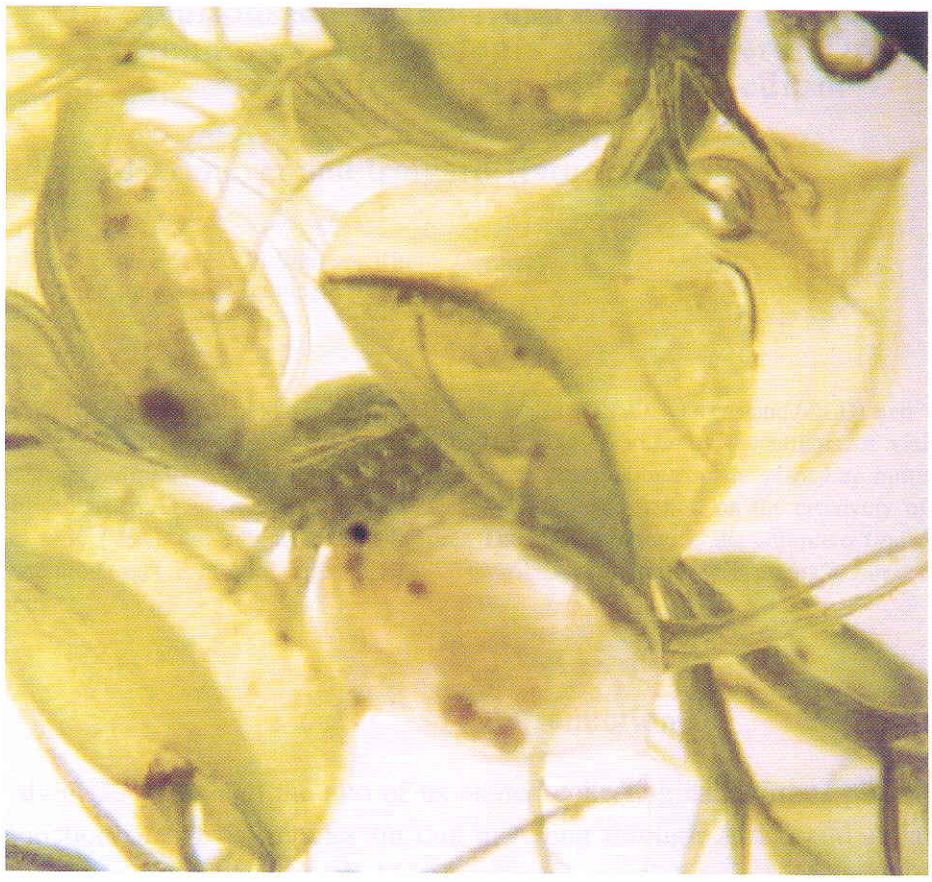

Figure 1. *Daphnia magna* on an *Aldrovanda* leaf (length of trap is approximately 4mm).

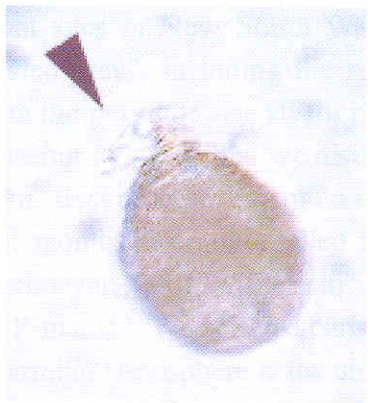

Figure 2. Testate amoeba from a culture of *Aldrovanda vesiculosa* from Girraween Lagoon, Northern Territory, Australia. The amoeba is approximately 20μm long, and the arrowhead indicates the extended pseudopodia.

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Observations on three Australian *Drosera* species of Sects. Arachnopus, Lanprolepis and Prolifera (Droseraceae), using digital video as medium

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Abstract: On several fieldtrips in New South Wales (NSW), Queensland (QLD) and Western Australia (WA), remote habitats and plants have been documented on digital video by the authors. A visit at the only known site of *Drosera schizandra* on Mount Bartle Frere (QLD), observations on the plant-bug mutualism on *Byblis filifolia* (Byblidaceae, WA) as well as *Drosera ordensis* (WA), and a report on the discovery of *Drosera hartmeyerorum spec. nov.* (WA), are subject of the lecture. The authors provide the complete tour 2001 as an entertaining, private produced CP-movie, called "FLEISCHIMANIA" (translated "CP-mania"), made for CP-laymen as well as experts.

Introduction

About 25 years ago the two of us started collecting carnivorous plants (CPs) as well as the production of private movies on our travelling through Africa and Asia. Ten years later we decided to combine the two hobbies, with the intention to provide entertaining videos on CPs and their habitats in Australia, Europe, and the Seychelles for interested CP-enthusiasts. Since 1999 we use digital equipment and a professional editing software for a better film quality and realised a new project on particular Australian CPs in March/April 2001. We visited exciting plant sites in New South Wales, Queensland and Western Australia, collecting 12 hours of "video-prey", including the recording of two excellent music events at Port Douglas (QLD), with the permit to use all for our film soundtrack. Fortunately we met our goal to find again an amazing *Drosera*, that we discovered in 1995 and that we could identify to be a species on it's own. Best accessories for an entertaining CP footage, to be edited after our return to Germany. Six months later, we called the resulting 60 minutes: "FLEISCHIMANIA" (Hartmeyer and Hartmeyer, 2001). "Fleischi" is a German nickname for CPs, so the title can be translated as "CP-mania". We selected parts from the movie for documentation, hoping to convey a bit of the charming atmosphere at the unique environments, and thus to increase the interest on the subject.

***Drosera schizandra* on Mount Bartle Frere (QLD)** All three *Drosera* species of the section Prolifera *(D. adelae, D. prolifera* and *D. schizandra*) grow inside the tropical rainforest of Northern Queensland. Until today pictures of the sites are rare, because all species grow at remote places, requiring a guide and proper preparations for the several kilometres long walk through the wilderness. Fortunately our friend Trevor Hannam from Cairns kindly agrees to guide us on an adventures trip to the wooded slopes of Mount Bartle Frere (1680 m), accompanied by leaches, spiders, snakes and last not least a cassowary. The resulting video sequence shows hand-sized *D. schizandra*, growing at the only known site under low light conditions on a layer of rotting leaves, covering the ground of a fascinating rainforest.

Plants and bugs from the Ord River region On our first visit to the Ord River region (WA) in1995, we found a single Sundew looking like a red *Drosera indica* (sect. Arachnopus) on a first glance, but showing distinct features on closer inspection. However, taking only video-pictures (Hartmeyer and Hartmeyer, 1995) was not sufficient for a scientific description of the species. For that reason we start a second approach to find the plant again in 2001. Actually we are very lucky and stumble upon a single plant on our first excursion in the area, however, we do not recognise the thrilling feature of the species during the first video shots. As we find numerous *Byblis filifolia* (Byblidaceae) inhabited by symbiotic *Setocornis species* (Miridae) on the same site, we decide to film this mutualism first and to return later for more shots of the deep red *Drosera*. To complete the documentation on the symbiosis, we also visit the sandstone formations of *Hidden Valley* to record the perfect adapted and - as far as we know - not yet scientific described *Cyrtopeltis species* (Miridae), living on *Drosera ordensis* (sect. Lamprolepis), documented also on our 1995 video. The pictures show clearly, how the bugs move freely on the trapping leaves, dropping their faeces as perfect nutrition for the predatory plant which provides sticking prey to feed on.

A new *Drosera* in sect. Arachnopus We return to the site with the deep red sundew and Irmgard discovers about 100 more of the nicely pink flowering and fruiting insect-eaters. On closer inspection, bright yellow dots in the centre of every individual catch my interest. I recall that also the single plant that we found six years ago, showed yellow parts (visible on video), which I mistook as sticking grass pollen. An attempt to remove the strange emergences proofs that they are part of the *Drosera* and no parasites or eggs of any insect, which could look similar. We decide to collect specimen for a scientific determination by Dr. Jan Schlauer (University Tuebingen, Germany), an international known CP expert and co-editor of Carnivorous Plant Newsletter. Knowing that a *Drosera*, carrying not only normal tentacles, but additional bright yellow stalked heads without mucilage must be something new. The purpose of that peculiarity is still just speculation. Actually some weeks later, Jan Schlauer confirms our opinion on the yellow emergences and kindly agrees to write the scientific description for CPN 30/4 (Schlauer, 2001). An interview with Jan Schlauer, who named the plant *D. hartmeyerorum spec. nov.* (sect. Arachnopus), was recorded in Germany and is part of the movie, which ends on that particular evening near Kununurra, with a colourful sunset at the plant site. "FLEISCHIMANIA" is a private production by the authors, copies are available in the English or German language and for several video systems.

Materials and Methods

Canon® MV20 Mini-DV camera, Sony® DHR-1000VC DV-recorder, FAST® DV-Master-PRO video-card, In-sync® Speed Razor® editing software, MS-Windows® NT 4.0 on 733-Pentium III computer

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Four seasonns of *Pinguicula ramosa* and its habitat of Mt. Koushin

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Abstract. Seasonal rhythms of *P. ramosa* and its habitat of Mt. Koushin are shown by VIDEO.

Pinguicula ramosa Miyoshi is endemic to Japan and is restricted to only a few mountains, north of Tokyo, including Mt. Koushin where the type locality of the species was discovered . Seasonal rhythm of *P. ramosa* and its habitat of Mt. Koushin, especially winter conditions, have not been well studied.

Full seasons of the species and Mt. Koushin were VIDEO-tape recorded from 1968 up to the present for analysis.

In late May, winter bulbs of *P. ramosa* begin to dehisce, grow leaves and already set flower buds.

In middle June, plants of the species are flowering. Flower angle is at 180° placed behind and rolled slowly to normal flower position. Inflorescences grow from horizontal to vertical direction and eight times as long as those at the beginning of the flowering. When the capsules get matured, the inflorescences are curved toward the cliff rock for the purpose of safty dispersal of seeds. The capsules with non-fertilized seeds do not get large and their inflorescences do not have any movement described above. Temperature is getting higher. At nearly final stage of flowering, plants of the species begin to produce some more leaves and capture preys. By middle August, they stop eating preys and complete their annual growth and finish seed dispersal. Their leaves turn yellowish color and plants set wilter bulbs. Some plants in warmer microclimate hold yellow colored leaves until October. In November, falling leaves of deciduous trees, winter bulbs go a little bit underground. In February, Mt. Koushin has deep snow over 1 m deep on the ground at the top down to Ginzan-Daira, former shrine office and to Tsurukame-Iwa Rock. Since steep cliffs of the habitat for *P. ramosa* are overhung, they do not deposit snow as well as being icebound and are very dry perhaps close to zero in water content. Dried dead leaves of the plants physically get erect and bundle winter bulbs. Winter bulbs in early winter contain much water and are, thus, hard. Then, they get soften in late winter. From late May to June, the cliffs of the habitat have much water and even drip water. Plants of *P. ramosa* may have correlationships between growth in this season with much water and new growth and flower numbers for next year. In July to the next April, the habitat is very dry and thus, the plants of *P. ramosa* are seemed to receive water from fog.

Acknowledgements

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Carnivory and parasitism in plants

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Abstract. Only a relatively small number of plant groups are capable of taking organic matter directly from other organisms. Plants that are capable of connecting to other plants and diverting nutrients from them using haustoria are known as parasitic plants. Plants that are capable of capturing animals and absorbing nutrients from them using traps are known as carnivorous plants. A comparison between these two syndromes is given in respect to mechanisms and classification, with reference to the difficulties in classifying the traps by mechanisms. The terms holo-carnivorous, hemi-carnivorous and pseudo-carnivorous are suggested for use in the discussion of carnivorous mechanisms. With parasitic plants it is clear that in natural habitats host and parasite coexist in an equilibrium that allows both of them to develop and to set seeds. An analogy between carnivorous and parasitic plants in this respect seems plausible once we consider the carnivorous plants as parasites of prey communities, not of individual prey. It is essential for the existence of any carnivorous plant species in natural habitats that the prey communities will co-exist with it in equilibrium. In addition, one should not rule out the possibility that in addition to the mutualistic relations that are known to exist between pitcher plants and insect communities that feed on them and serve as prey, mutualistic relations may also exist between insects and other carnivorous plants.

Introduction

Most plants are autotrophic, producing their own organic substances thanks to their ability to photosynthesize sugars. Only a relatively small number of plant groups can take organic matter directly from other organisms. One important group is the carnivorous plants that can take animal matter; the other group is the parasitic plants that can take nutrients from other living plants. Both groups developed special organs that allow these capabilities.

The understanding of the mechanisms of parasitism and carnivory has reached some peaks in recent years, yet much is still to be studied in these two interesting plant groups. The aim of the present paper is to compare the two groups, which seem to have much in common.

Definitions

Parasitism in plants is defined as the ability of a plant to divert water and nutrients from other living plants by a special organ, the **haustorium** (Kuijt, 1969). Plants that are capable of connecting to other plants and diverting nutrients from them using haustoria are known as parasitic plants (PP).

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Carnivory in plants is defined as the ability of a plant to capture animals and to use them for the plant nutrition, by a special organ, the ***trap*** (Juniper *et al.*, 1989). Plants that are capable of capturing animals and absorbing nutrients from them using traps are known as carnivorous plants (CP).

Both definitions are not satisfactory, having many exceptions. This is natural because any such definition is arbitrary, based on human perception of plants rather than on clear biological boundaries between plant groups. Such biological boundaries simply do not exist. Nevertheless, for our convenience, these general definitions allow to examine relatively small groups of plants that share some unique series of characters.

Frequency of occurrence

Both carnivorous and parasitic plants are relatively rare in nature, but they are often domesticated either purposely as ornamentals, in the case of carnivorous plants (some of which are massively grown in culture), or accidentally as weeds, in the case of parasitic plants (some of which threaten agricultural productivity).

Polyphyletic evolution

The carnivorous syndrome and also the parasitic syndrome are polyphyletic, each has developed independently in various non-related plant groups, nevertheless they show similar organs and seem to function in similar manners. For example, adhesive traps can be found in *Drosera* and *Pinguicula* that belong to two non-related plant families. Likewise, pitchers can be found in *Cephalotus*, *Sarracenia* and *Nepenthes*, and moving traps in the aquatic *Aldrovanda* and *Utricularia*, both latter cases showing a concurrent occurrence of similar trapping devices in non-related plant families.

With parasitic plants the situation is very similar. Root parasites developed in e.g. the Rafflesiaceae, Balanophoraceae, Hydnoraceae and Orobanchaceae that are non-related plant families. Likewise, twining leafless parasites can be found in the non-related genera *Cuscuta* (Convolvulaceae) and *Cassytha* (Lauraceae).

The Scrophulariaceae in relation to CP and PP

An interesting link between CP and PP is provided in the Scrophulariaceae. This plant family that exhibits many autotrophic plants is also closely related to both Orobanchaceae and Lentibulariaceae. Whereas the latter is know as a family of carnivorous plants, (*e.g. Utricularia, Pinguicula,* and *Genlisea*) the former is a family of holoparasitic plants (*e.g. Orobanche, Cistanche,* and *Epifagus*). Parasitic plants in the Scrophulariaceae and Orobanchaceae families have been traditionally depicted as forming a linear evolutionary series beginning with hemi-parasitism (*e.g. Striga hermonthica*) and ending with holo-parasitism (*e.g. Cistanche spp.*). The Scrophulariaceae genera *Lathraea* and *Hyobanche* have been viewed as transitional links between the hemi-parasitic members of Scrophulariaceae and the strictly holo-parasitic habit of the Orobanchaceae (Young *et al.*, 1999). But it also seems that in the Scrophulariaceae parasitism and carnivory developed concomitantly. The extreme case in this respect is found in the genus *Lathraea.* Plants of this genus are holoparasitic, but they also carry special small utricles that may serve as traps. The function of these organs is not yet understood, yet it was suggested that *Lathraea* represents a plant that had both parasitic and carnivorous capabilities (Juniper *et al.*, 1989).

Classification of traps

The carnivorous plants are basically classified according to their taxonomic status. In addition we often classify them by their trapping mechanism, which may vary even in a

single plant family. Thus, flypaper traps can be found in both Droseraceae and Lentibulariaceae, but these two families also exhibit other trapping mechanisms, such as snap-traps in the Droseraceae and suction traps in the Lentibulariaceae (Lloyd, 1942).
Classifying the traps on the basis of their functional principle is difficult. In Sarraceniaceae, for example, some *Sarracenia* species secrete a digestive fluid and are fully carnivorous, other species depend on external sources of water and on digestive enzymes secreted by microorganisms or by insect inquilines for prey breakdown and digestion (Hepburn *et al.*, 1927; Joel and Heide-Jorgensen, 1985), and some *Heliamphora* species seem not to benefit at all from their traps in terms of digested animal matter (Juniper *et al.*, 1989). More so, plants like *Roridula* do not have any digestive capabilities, nor do they have digestive gland, but they developed mutualistic relations with a hemipteran insect that helps in transmitting nutrients from the prey to the plant. This insect consumes the prey that is captured by the secretion on the stalked glands of *Roridula*' s leaves, and the faecal nitrogen of the insect is then taken up by the leaves (Ellis and Midgley, 1996). We can see that in spite of the lack of a digestive system in *Roridula*, it can still be considered carnivorous because it fits into the CP definition, i.e. capable of capturing animals and absorbing nutrients from them in traps.

Similarity between parasitic and carnivorous plants

Are there any similarities between these two plant groups? Indeed both have alternative feeding organs, the trap and the haustorium, and both are able to use organic substances from other organisms. All carnivorous plants are photosynthetic, i.e. they are green plants, able to produce organic substances of their own through photosynthesis. This is also true for many parasitic plants.
Nevertheless, some highly specialized parasitic plants are devoid of any photosynthetic capability. In fact, parts of the photosynthetic genome in these plants are missing or altered (Young *et al.*, 1999). As a consequence, these obligatory parasitic plants are classified as ***holo-parasites***, since they are totally dependent on their hosts for organic matter. On the other hand, parasitic plants that are able to supply themselves with at least some organic matter through photosynthesis, having a functional photosynthetic genome, are classified as ***hemi-parasites*** (Kuijt, 1969). This nomenclature is very convenient, though sometimes controversial.
In a similar manner one may also classify the carnivorous plants according to their carnivorous status. Carnivorous plants that trap animals, digest them using their own digestive glands, and absorb nutrients from the prey may be classified as ***holo-carnivorous***. Clear examples for this group are *Dionaea muscipula and Drosera rotundifolia.* Carnivorous plants that have traps and are capable of absorbing digested animal matter, but do not secrete digestive enzyme by themselves, may be classified as ***hemi-carnivorous***. An example for this group is *Roridula spp.* And those plants that do not exploit animal matter for their nutrition but have organs that are homologous to known traps, may be classified as ***pseudo-carnivorous*** .
Parasitic plants can also be classified as obligatory if their development is fully dependent on nutrient supply from a host. A similar classification is irrelevant for carnivorous plants simply because there is no single carnivorous species that is known to fully depend on the supply of animal matter. Nevertheless there are some carnivorous plants that are known to benefit a lot from carnivory, in term of growth rates and seed set (Darwin, 1875), while for other species the contribution of animal matter is not significant.

Specific organs

As stated above, carnivorous plants have digestive surfaces, and parasitic plants have haustoria. Both organs allow the transfer of nutrients from other organisms that serve as sources of nutrients. The digestive glands in carnivorous plants vary from a small organ containing a few cells (in *Utricularia* and *Cephalotus*) to a multicellular

multilayered gland (in *Drosophyllum, Triphyophyllum* and some species of *Nepenthes*). Despite the differences, the digestive glands and also other digestive surfaces, like the pitcher epithelium of *Sarracenia*, show a common architecture that is composed of the same components in almost all carnivorous plants: glandular cells on top, and endodermoid cells mediating between the glandular cells and the leaf tissues (Joel, 1986). Usually conductive elements are also present, allowing the transport of digestion products from the glands to other plant organs.
Similarity can also be found in haustoria structure in the different parasitic plant groups. Haustoria are principally composed of the same components in almost all parasitic plants (Kuijt, 1969), and develop in similar manners: intrusive cells penetrate host tissues, and then the intrusive body develops conductive tissues that connect directly to the conductive tissues of the host (Joel *et al.*, 1998). This is true for root parasites, twining parasites, and mistletoes.
. Clearly, the digestive surface in CP, which is usually composed of numerous digestive glands, serves as an active transport organ that absorbs animal matter from the trap cavity (or from the digestive pool). This structure actively controls the transfer of digested substances to the plant conductive system. The haustorium in parasitic plants is likewise capable of absorbing water and nutrients from neighboring host tissues during its early development. However, it seems that once the haustorium matures, it usually serves as a passive bridge rather than as an active sucking organ (Joel *et al.*, 1998). This difference between haustoria and digestive glands is intrinsic in the basic nature of these two syndromes. While the parasitic plants divert nutrient supply from a living organism to which they connect, the carnivorous plants digest and absorb organic matter from non-living organisms. In the former case the parasitic strategy is based on the formation of a strong 'sink' that drives nutrients from the host. In the latter case the carnivorous plant cannot manipulate the animal to supply it with nutrients; it simply takes the nutrients that build the body of the prey by external digestion and active absorption.

Perception of chemical stimuli

Unlike almost all other higher plants, seed germination in obligate root parasites depends on receipt of a chemical signal from the roots of host plants. These parasitic plants are totally dependent on a specific association with a host that provides them with nutrients and water, so the chemical recognition system ensures that germination starts only when suitable host roots are available in the immediate vicinity of the parasite seed. A similar dependence of seed germination on an external chemical stimulus does not exist for carnivorous plants. However, the onset of digestive activity is often triggered by a chemical stimulus that originates in the captured prey (Juniper *et al.*, 1989). In both cases, very low stimulant concentrations are needed. In *Dionaea* a chemical stimulus leads first to the development of cuticular gaps in the digestive glands (Joel *et al.*, 1983), and then it also elicits the secretion of the digestive fluid into the trap cavity (Robins, 1976). The chemical recognition of a prey inside the trap ensures that the plant spends its digestive resources only when suitable prey is available in the immediate vicinity of the digestive surface.

Attachment to the alternative source of nutrition

The first step both in a parasitic activity and in a carnivorous trapping of prey is the attachment of a specialized plant organ to the source of alternative food. In the parasitic plants this is done by the attachment organ, a unique structure that adheres to the surface of the host plant, usually with the aid of a special anchoring secreted substance. This strong anchoring allows the parasite to push the intrusive cells into host tissues without disconnecting from the host (Joel and Losner-Goshen, 1994). In the carnivorous plants the attachment is done by the trap that captures the prey and retains it, usually with the aid of a liquid that allows retention and close contact for later digestive

activities. However, while the parasites take nutrients from living organisms, the carnivorous plants kill their prey before nutrients can be taken. Thus, enzymatic activities in carnivorous plants are responsible for the digestion of prey tissues (Juniper et al. 1989), whereas enzymatic activities of parasitic plants allow the intrusion into living host tissues (Losner-Goshen *et al.*, 1998).

Nutrient transfer

In both carnivorous and parasitic plants the taken nutrients are transferred to the plant sinks, *i.e.* to the developing leaves, roots, flowers, etc. While it is known that the incoming nutrients are first metabolized in the traps and only then transferred to the vegetative or reproductive apices in carnivorous plants (Juniper *et al.*, 1989), it is not yet clear whether the nutrients absorbed by parasitic plants are first metabolized in the haustoria, or, as it may also be the case, the two mature organisms, the parasite and its host, co-exist connected to each other. In this case the parasite serves, in physiological terms, just as an additional sink in the "united organism".

CP-prey and PP-host relations

The above discussion brings us to some basic characteristics of parasitism in plants. There are two important stages in plant parasitism. The first is characterized by intrusion of the young parasitic haustorium into host tissues, bypassing or neutralizing the host defense mechanisms of the host (Joel and Portnoy, 1998). This is the intrusive developmental stage. Then the haustorium matures, and the host and parasite coordinate their growth in such a manner that leads to precise alignment of the conductive tissues of the parasite with those of the host (Joel, 2000). This is the coordinated developmental stage. The parasite clearly benefits from these relations, being nourished by its host. This is essential at least for holo-parasites. Though nothing is known on possible benefits for the host from these relations, it is clear that the two partners eventually coexist in an equilibrium that allows both of them to develop and to set seeds. Damage is caused to the host only in disturbed systems (like agricultural fields) where this equilibrium is broken, not in undisturbed natural habitats. Clearly the parasite does not impose any threat on the existence of natural host species communities.

With carnivorous plants the situation looks different; nevertheless one can make some analogy between carnivorous plants and parasitic plants in this respect, once we consider the carnivorous plants as parasites of prey communities. Although prey capture is usually fatal for any individual prey, the community of any particular animal species is never endangered by carnivorous plants. Whatever number of individuals the animal community loses, it is essential for the existence of the carnivorous plant species that the prey community will co-exist with it in equilibrium.

The relations between a carnivorous plant and its prey community may differ, though, in different systems.

With some pitcher plants it is now clear that their relations with some prey communities are mutualistic rather than parasitic. Insects benefit from pitcher nectar that serves for their nutrition in habitats where sources of floral nectar might be uncommon. At the same time the insects pay the plants in a small portion of their community which is 'sacrificed' as prey and consumed by the plants, which grow in nutrient-poor soils (Joel, 1988). It seems that nobody has ever looked into the influence of insect trapping by non-pitchered CP on the insect community in natural habitats. Other mutualistic relations may be discovered. In any case one may put forward the hypothesis that carnivorous plants can reduce the size of an insect community to the extent that reduces intra-specific competition within this community. By this, the CP would probably not differ from other predators in a balanced habitat.

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Global carnivorous plant diversity - Insights and incentives

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Although the taxonomy of some notoriously "difficult" species or aggregates is not settled yet, and although systematic ranks are not necessarily equivalent in different phylogenetic contexts, carnivorous plant diversity can be assessed systematically by an analysis of the global distribution (presence or absence in floristic regions and provinces) of species, sections, subgenera, genera, and families, which adjusts artifacts from differing taxonomic interpretations (Schlauer, 2000). These diversity data can be evaluated from various viewpoints.

Phytogeography: carnivorous plant taxa can be classified according to their distribution pattern. Only few taxa display a pantropical or cosmopolitan distribution, and even these can be referred to more localized groups if their closest relatives are compared. Endemisms frequently indicate geographic origins of superordinate taxa. Thus, primary and secondary centers of diversity can be identified.

Evolution/Systematics: phylogenetic processes can not only be deduced from the mutual similarity (in morphological or molecular characteristics) of extant taxa but also from geographic distribution patterns, because evolution takes place in both time and space. Vast disjunctions are the exception rather than the rule, and spatial analysis can be refined considerably by considering distribution patterns at progressively higher systematic ranks. With this method general trends and obvious exceptions in both geographic distribution and distinguishing characteristics can be identified. By comparison of geographic origins and present day geographic distribution, lineages can be reconstructed even in cases where time courses are unknown and fossils are missing or scant, like in many groups of carnivorous plants.

Conservation: both the alpha diversity (number of taxa present in a given phytogeographical unit; a quantitative measure of diversity) and the degree of endemism (number of endemic taxa vs. total number of taxa present; a qalitative measure of diversity) provide important clues to the reasonability of conservation efforts in a given phytogeographic unit. Arbitrary political boundaries (that are still the effective limits of legislation) are almost meaningless in a global conservation perspective.

General: centers of diversity also display a high degree of endemism, and this trend is frequently paralleled also at higher taxonomic ranks. The 6 primary regions (from a total of 35 regions distinguished here) of high species diversity and endemism are SW Australia (*Drosera*), Malesia (*Nepenthes*), Mexico (*Pinguicula*), the Guayana Highlands and the Brazilian Uplands (*Utricularia* and *Genlisea*), and N Australia (*Utricularia*). 7 Province endemics (from 152 provinces distinguished in total) at generic and familial rank are present in the Cape (*Roridula*, Roridulaceae), the Guayana Highlands (*Heliamphora*), N California and adjacent Oregon (*Darlingtonia*), the Atlantic Coastal Plain of SE N America (*Dionaea*), the SW Mediterranean (*Drosophyllum*, Drosophyllaceae), Upper Guinea (*Triphyophyllum*, Dioncophyllaceae), and SW Australia (*Cephalotus*, Cephalotaceae). *Sarracenia* is almost endemic in SE N America, only one species (*S. purpurea*) has reached a wide distribution further N, reflecting a rapid, postglacial range extension. *Byblis* (Byblidaceae) is almost restricted to Australia, only one species (*B. liniflora*) is known to extend to E Malesia. The remaining carnivorous plant genera, of which only *Aldrovanda* is monotypic (but remarkably represented by several different species in the fossil record), are more widespread. Their infrageneric diversity and distribution are discussed in more detail in the oral presentation.

Reference

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Evolution of carnivory in the Lentibulariaceae: considerations based on molecular, morphological, and physiological evidence

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ABSTRACT. Data from recent molecular phylogenetic studies on the Lentibulariaceae and on related families in the Lamiales are discussed in the light of a potential scheme for the evolution of carnivory in this plant family. It is suggested that all 3 extant genera of the Lentibulariaceae (*Pinguicula*, *Utricularia* and *Genlisea*) evolved from a common, more or less aquatic (submerged under a very thin film of water or wetted regularly by rain or fog), and rootless (no central root system) rosetted ancestor. This ancestor would not be related to the Lamiales procarnivorous families, Martyniaceae and Byblidaceae. It might have been making use of sessile glands on its leaves (a potential speciation from an ancestral gland type shared with the rest of the Lamiales) to absorb nearby nutrients and secrete enzymes to digest surrounding organic matter and increase nutrient availability next to its leaves. With the end result of better securing catches (and thus becoming carnivorous) and limiting water losses on traps, 2 strategies have been developed. One has consisted in producing a novel gland type, a stalked gland, that secretes mucilage to glue preys and prevent dehydration, and that generates a bowl (curling effect) under the preys to retain digestive fluids. *Pinguicula* has probably evolved according to this pattern. The nearest ancestor of our *Pinguicula* sampling was living in the northern hemisphere and was producing an hibernaculum to resist frost. This hibernaculum has subsequently evolved into a drought resistant form (and in some cases later disappear altogether) in the Mexican taxa. A larger sampling is likely to shed light on a more ancient past of this genus. *Utricularia* and *Genlisea* are suggested to have worked out a separate strategy towards carnivory whereby they have closed (more or less tightly) their trap-leaves and inverted their tropism to generate upside-down bladders that suck and filter food permanently from their aqueous environment (anything from already dissolved matter to microscopic organisms). Both bladder-bearing genera developed different ways of securing their catches. *Utricularia* traps have a proper door and doorstep system of closure while *Genlisea* traps have a long neck and 2 arms next to a permanently opened trap mouth. *Genlisea* neck and arms inside surfaces are lined with downwards-pointing hairs that force the flow of prey to only migrate towards the digestive cavity and never escape. Early diversification of *Utricularia* and *Genlisea* is thought to have taken place in the southern hemisphere.

Introduction

The evolution of carnivorous structures in plants represents one of the most complex and most fascinating enigmas in plant sciences. Recent attempts to find their origins with genetic tools have been made on several groups of carnivorous plants such as the Droseraceae and related families (Albert *et al.*, 1992), Nepenthaceae (Meimberg *et al.*, 2000), and the Sarraceniaceae and related families (Bayer *et al.*, 1996). However, the Lentibulariaceae family that makes up nearly half of the total number of carnivorous plant species had so far never been thoroughly investigated.

Lentibulariaceae comprise 3 carnivorous genera, *Pinguicula*, *Genlisea* and *Utricularia*. All 3 genera share characteristics in flower structure (zygomorphic flowers with a spur)

but exhibit widely different carnivorous habits. *Pinguicula* species have the simplest trapping device. Their leaves possess sticky glands and they catch prey by acting like flypapers. Because of this apparent simplicity of action, it has been widely suggested that they could represent an ancestral form that led to the development of the more complex-looking *Utricularia* and *Genlisea* suction type traps (Taylor, 1989). In this respect, *Genlisea* species have been considered to be evolutionary intermediates between the 2 other genera since they generate bladder type traps but do not posses a sophisticated door system to secure any catch like *Utricularia*. Along the same line of thinking, Byblidaceae and some Martyniaceae (called procarnivores in this study) have been suggested to be descendants of an even more ancestral form since their leaves act as flypaper traps that cannot digest their prey (Lloyd, 1942, Hartmeyer, 1997, 1998; Meyer-Rice, 1999; Wallace and McGhee, 1999). Affinities of Lentibulariaceae and procarnivores within Lamiales are suggested by morphology and were supported by broad scale molecular phylogenetic analyses (Albert *et al.*, 1992; Chase *et al.*, 1993; Soltis *et al.*, 2000; Albach *et al.*, 2001). However, exact relationships remained uncertain.

Ever since scientists have unveiled the heredity of the DNA molecules that are contained in each living cell, they have tried to access the information it contains to better understand the past history of modern life on earth. The recent complete sequencing of the human genome has been a giant step in this direction (Lander *et al.*, 2001; Venter *et al.*, 2001). It revealed that all of the genes that act to maintain our body living barely make up 1.5 % of our total DNA and that the rest is an immense junk yard of non-functional genetic information that has accumulated over billions of years of evolution like geological layers in an archeological site. But what can they tell us? How were they deposited? How has new DNA information been created? These are questions scientists still cannot answer fully. It is still out of the question to conduct a complete genome sequencing of every living organism. Current technologies (including computer technologies) do not allow it. What is possible at the moment is to look at small, carefully chosen, portions of the full genome whose variations among sampled individuals have enough statistical significance to draw conclusions.

Over the past three years, we have attempted to generate DNA sequence data of a large group of species of the Lentibulariaceae and related families within the Lamiales (Müller *et al.*, 2002a and b). Our DNA target has been the widely used chloroplast gene *matK* (Neuhaus and Link, 1987; Hilu and Liang, 1997) and the rarely used adjacent intron sequences (trnK intron). New computer software has been designed to facilitate alignment, phylogenetic analysis and analysis of rate heterogeneity (Müller, 2000; Müller *et al.*, 2002b; Müller, 2002). Besides molecular work, a lot of progress has been made to understand the biology of Lentibulariaceae. For example, Barthlott *et al.*, 1998 showed *Genlisea* to be trapping protozoans. To understand the evolutionary diversification of the Lentibulariaceae, we attempted to link our molecular phylogenetic data to a careful analysis of morphological features, in particular the differences/similarities in gland anatomy (Müller *et al.*, 2002a, b). The present article will review some of the conclusions of this work and will outline one possible scenario for the evolution of members of the *Lentibulariaceae*.

Materials and Methods

Information concerning the methods used for DNA extraction, amplification and sequencing can be found in Müller *et al.* (2002a, b). Computer software used and developed for analysing the data are also described in the above manuscripts.

A set of 32 *Utricularia* species, 6 *Genlisea* species and 27 *Pinguicula* species with representatives from most known major sections of these 3 genera has been selected. Sections were derived from Fischer *et al.* (2000), Casper (1966), Legendre (2000) and Taylor (1989). In addition, species belonging to 22 related Lamiales genera were included in the data set. Voucher information and GenBank accession numbers can be found in Müller *et al.* (2002a, b).

Results

Phylogenetic relationships between procarnivorous taxa and Lentibulariaceae in Lamiales Parsimony and Likelihood analyses of matK and trnK-intron sequence data clearly support the current grouping of *Utricularia*, *Genlisea* and *Pinguicula* in one family (Müller *et al.*, 2002a), so that all three genera share common ancestry (*i.e.*, Lentibulariaceae are monophyletic). There is strong evidence that the evolution of true carnivory in Lentibulariaceae is independent from that of procarnivory in Byblidaceae and Martyniaceae, *i.e.*, preadaptations to carnivory were acquired at least twice independently in this angiosperm order. However, only limited statistical support was obtained for the respective phylogenetic relationships as inferred from *matK* and *trnK* sequences. Additional Molecular markers are now under scrutiny to better resolve this important issue. Earlier results obtained by Albert *et al.* (1992) with the chloroplast gene *rbcL* suggested that the procarnivorous *Byblis* might be the closest relative of the carnivorous Lentibulariaceae However, this conclusion was based upon a very small Lamiales sampling and not supported statistically.

The distribution of morphological characters supports the phylogenetic relationships reconstructed with molecular data. This becomes especially evident when looking at the structure of the glands of the traps of carnivorous and procarnivorous plants. As shown in Figs. 1 and 2, the carnivorous/procarnivorous species of the Lamiales are the only insect trapping plants in which gland stalks are made of stacks of layers of single cells. While this may suggest common ancestry, significant differences among the various members do not support such a conclusion and it is just as likely that the formation of these simple glands is easy enough to have occurred, or have been modified, several times in the evolution of the Lamiales. The glands of the traps of the members of the Lentibulariaceae are all positioned on a single epidermal cell, while those of Byblidaceae and Martyniaceae develop on a minimum of two epidermal cells (Juniper *et al.*, 1989). The digestive glands of the members of the Lentibulariaceae are all attached to sap vessels unlike any other gland of the Byblidaceae and Martyniaceae. Finally, the central cell of the digestive glands of the Lentibulariaceae (endodermoid cell) possesses physiological characteristics (thickened cell wall/cuticle, endoplasmic reticulum organisation, accumulation of fluorescent substances) that have not been described in any other member of the Lamiales (Juniper *et al.*, 1989; Legendre, 2000). All of this supports the hypothesis of an independent evolution of the incomplete carnivorous syndrome in the procarnivores.

It is interesting to note that carnivorous plants are the only angiosperm species that have glands (digestive ones) that are linked to sap vessels (Juniper *et al.*, 1989). Lloyd (1942) argued that the spongy tissue on which *Byblis* glands sit may act as a conductive medium for the rapid absorption/release of substances. No experimental evidence has been provided. It is, therefore, possible that the linking of glands to sap vessels is a necessary condition for effective digestion and absorption of organic matter by speeding up the transport of enzymes and nutrients.

Phylogeny of Lentibulariaceae The analysis of *trnK* sequences from 32 *Utricularia* species, six *Genlisea* species and of 27 *Pinguicula* species, revealed that Lentibulariaceae species cluster into two separate groups (Müller *et al.*, 2002a). One comprises the two bladder trap plants (*Utricularia* and *Genlisea*) and the other one is constituted by the flypaper trap genus, *Pinguicula*. *Pinguicula* is, therefore, a sister group of the two former genera. Our data show that all three genera are monophyletic. Each of these infrafamiliar relationships receives maximum statistic support.

To our great surprise, the rate of mutation of *matK* and the noncoding *trnK* intron sequences was found to be much higher in *Utricularia* and *Genlisea* than in *Pinguicula* (Müller *et al.*, 2002b). Even when compared to other genera in flowering plants, *Utricularia* and *Genlisea* still exhibited the fastest evolutionary rates in *matK*. In *Pinguicula*, this gene was found to evolve just as fast as the fastest previously known rate observed in some parasitic plants. This increased DNA evolutionary rate of *Utricularia* and *Genlisea* was confirmed when other genes from other plant genome pools were studied (Müller *et al.*, 2002b).

An analysis of gland structures supports the relationships found with molecular data. All 3 genera share one type of gland. This gland is the sessile gland of *Pinguicula*, the bifid/quadrifid gland of *Utricularia* and the digestive gland of *Genlisea* (Fig. 1). It is made of an epidermal cell on which sits an endodermoid cell that supports a varying (always a multiple of 2) number of head cells. The number and shape of the head cells varies among genera and species but also on one given trap (Lloyd, 1942, Casper, 1966, Juniper *et al.*, 1989). This conserved gland structure also serves a common function since, in all 3 genera, it is involved into the secretion of digestive enzymes and the absorption of nutrients. It does not secrete mucilage and is invariably attached to some tracheid element (xylem and/or phloem).

Pinguicula leaves harbour another type of gland, a pedunculate gland, which is specific to this genus. Compared to the digestive glands, this gland exhibits an additional elongated stem cell that elevates the endodermoid and radiating head cells from the surface of the epidermis. It also sits on an enlarged epidermal cell (reservoir cell) that further elevates the whole structure. This epidermal cell is not linked to a tracheid element but is in contact with numerous other cells via plasmadesmata (cell to cell communication structure). The pedunculate gland of *Pinguicula* is involved in mucilage production, enzyme and water secretion, and prey capture but does not seem to be involved in nutrient absorption (reviewed in Legendre, 2000). Its enzyme and water secretory mechanism is unique to the Lamiales in the sense that it is a one-off process whereby the head cells secrete their entire content once stimulated by a prey to then engage into a self-destructive mechanism. Thus, this gland can only secrete once in its lifetime. The stalked gland of *Pinguicula* secretes a smaller set of hydrolytic enzymes than the sessile glands (lack of leucine aminopepdidase for example). When similar enzymatic activities are present in both gland types, their intracellular distribution may vary (RNase activity for example–reviewed in Juniper, 1989). Even though *Utricularia* and *Genlisea* do possess mucilage-producing glands, these have a totally different anatomy (Fig. 1). The *Utricularia* mucilage glands are situated close to the entrance of the door of the trap (outside of the trap, i.e., back side of the leaf, see below) where they allow a good sealing of the trap door. Their stem is an elongated epidermal cell (not an additional stem cell). The mucilage producing glands of *Genlisea* are also situated on the outside of the trap contrary to *Pinguicula* species (if *Pinguicula* species have mucilage glands on the under side of their leaves, they also contain digestive glands on that same leaf surface; see *P. gigantea* for example). They comprise an epidermal cell, a small intermediate cell and a small, round head cell.

Finally, *Utricularia* possesses some glands that are unique to their genus. *Utricularia* traps harbour a type of small gland on their outside surface. It is made of an epidermal cell, an intermediate cell and one head cell. Interestingly, this gland resembles the mucilage gland of *Genlisea* in both shape and position even though its function has been hypothesised to be different (absorption of nutrients in immature traps, Lloyd, 1942). *Genlisea* harbour a large variety of trichome hairs inside their traps and at the edge of their trap arms to clip them. *Pinguicula* and *Utricularia* also express a large variety of hairs but none are located among the digestive glands.

Phylogeny of *Pinguicula* An analysis of the genetic relationships among *Pinguicula* species generated a very surprising result (Müller *et al.*, 2002a). Based on our current sampling, *Pinguicula* species can be divided into 2 separate subgroups. One (called group A) constitutes all of the temperate *Pinguicula* spp. minus *P. alpina* which belongs to the other group (called group B) that includes all Mexican/central American/Cuban species. Even though hybrids between *P. alpina* and *P. vulgaris* (temperate species) have been reported (Casper, 1966), the authors have not succeeded in hybridising these 2 species, as expected if the above genetic grouping is correct. In contrast, many temperate and Mexican species have been reported to hybridise well with members of their own clade (group of species sharing common ancestry). The temperate species (group A) are subdivided into 2 subgroups. One comprises *P. leptoceras* and *P. poldinii* while the other subset contains the remaining species. These 2 groups are differentiated by a large number of genetic differences. However, species of the large group (comprising *P. vulgaris, P. vallisneriifolia, P. grandiflora, P. macroceras, P. corsica, P. longifolia, and others*) exhibit no significant differences in

DNA sequences of trnK. In contrast, the Mexican species are separated by longer branches. *Pinguicula alpina* is sister to the Mexican clade. Within this clade the basal branching taxa are *P. filifolia*, *P. gracilis* and *P. rotundiflora*. Then follows as separate clusters *P. ehlersiae* and a clade consisting of *P. sharpii*, *P. gigantea* and *P. aganta*. Finally, the most terminal clades consist of *P. moctezumae* and *P. moranensis* on one hand, and *P. emarginata* and *P. rectifolia* on the other. This phylogeny of *Pinguicula* based on molecular data contradicts the current subclassification of the genus into 3 subgenera, *Isoloba, Temnoceras* and *Pinguicula*. Even though our sampling of species allowed wide representation of most previously defined sections of *Pinguicula* (Casper, 1966; Legendre, 2000), we are currently adding crucial species that will be needed to better understand the evolutionary history of the genus.

All *Pinguicula* species harbour similar sets of glands. They, however, exhibit different growth characteristics. Casper (1966) proposed to distinguish the temperate growth type (forming an hibernaculum) from the tropical growth type, both being subdivided into homophyllous and heterophyllous subgroups depending on whether the plants generate only one or 2 types of leaves during the year. This classification does not match the results of our cladistic analysis based on DNA sequences (Müller *et al.*, 2002a). Both group A and B contain temperate and homophyllous species. Rather, species of groups A and B differ in their time of flowering. All species of group A flower (create flower buds) in the spring after producing a first set of carnivorous leaves while most species of group B, and at least all of the basal ones (*P. alpina*, *P. rotundiflora* and *P. gracilis*), generate flower buds from their winter resting bud before the onset of production of carnivorous leaves. However, this floral characteristic has been poorly recorded on wild Mexican specimens and has sometimes been faulted by observations made on greenhouse grown plants. Further research is needed to validate this observation. We have seen no morphological trait that can associate *P. leptoceras* and *P. poldinii* while distinguishing them from all other European temperate species. The grouping of Mexican species based on their genetic characteristics is as difficult to link to any physiological structure previously used by taxonomists. Further research and a larger sampling will be needed to find morphological characters supporting the above grouping of species.

Phylogeny of *Utricularia* *Utricularia* phylogeny was found (Müller *et al.*, 2002a, b) to support the latest intrageneric classification of the genus by Taylor (1989) that was based on morphological data, predominantly from the traps. Pollen morphology was studied by Lobrean-Callen *et al.* (1999) and seems to be in line with the other phenotypic characters. However, in contradiction to Taylor's concept, our results indicate that section *Polypompholyx* is an integral part of the genus *Utricularia*. As already suggested by Lloyd (1942) and Taylor (1989), this section was found to represent an ancestral lineage in the genus, along with section *Pleiochasia* (called group A). Following Taylor's division of *Utricularia* into terrestrial, epiphytic or aquatic species, all so-called epiphytes (called group B' here) and aquatic species (called group B) were found to form separate clades that evolved from within a group of terrestrial species (group A, for sections *Polypompholyx* and *Pleiochosia*, and group A' for the remaining sections).

Trap functioning and overall gland structures have previously been described as being conserved within the genus *Utricularia* (Lloyd, 1942). The shape of the head cells of the digestive glands has, however, been reported to vary among species. These variations have been used successfully as a taxonomic character (Taylor, 1989). The decorations on the outside of the trap (such as ventral and mouth appendages) also vary among species and have been used at a lower taxonomic level in agreement with our data. Heslop-Harrisson (1975, 1976), has suggested that aquatic *Utricularia* species are the only ones to bear active traps and thus represent the most evolved/advanced members of the genus. Taylor (1989) did not support the idea that *Utricularia* traps can be divided into active and passive ones. He suggested that European aquatic *Utricularia* could be the most advanced form of the genus since they do not produce rosettes and make the largest use of stolons. These claims are, however, not supported by our data (Müller *et al.*, 2002a).

Even though trap functioning is conserved within the genus *Utricularia* (Taylor, 1989), the position of the traps on the plant varies and has been used to distinguish groups of sections of this genus (Taylor, 1989). Our data (Müller *et al.*, 2002a) support this observation. The central stem of *Genlisea* species and the species of the basal sections of *Utricularia* (group A) forms a dense rosette of leaf-like (see later for a discussion of the organs in *Utricularia*) organs and traps. In contrast, species of group A' generate traps on either the leaf-like structures or on stolons but never from the central rosette (main stem). In group B', members of the so-called epiphytes bear traps on the main stem of their rosettes, while the so-called aquatic group members (group B) bear traps on the leaf-like structures only. The branching of the photosynthetic organs is a feature that distinguishes most species of groups A/A' from those of groups B/B' even though it has not been previously used taxonomically. With the exception of *U. sandersonii* and related species, all members of groups A/A' possess leaf-like structures that contain only one undivided vein. Conversely, with the exception of *U. subulata*, all members of group B/B' harbour leaf-like structures whose veins fork many times, the laminae either joining the veins as in the so-called epiphyte group (group B') or leaving them separate as in the so-called aquatic group (group B).

Discussion

With the above data, we will now attempt to discuss a scheme for the evolution of the carnivorous syndrome in the Lentibulariaceae. Such a scheme assumes that the simplest evolutionary path has been followed. It is highly dependent on the amount of information available at the moment and may be faulted by past relatives that may have disappeared. Unfortunately, the fossil record of our group of plants is extremely poor. Our evolutionary scheme is presented as an educated guess to be used for future studies and discussions on this topic.

Physiological characters that are shared by all *Pinguicula*, *Utricularia* and *Genlisea* species are most likely so because they were present in their common ancestor. Examples of independent and convergent evolution of specific morphological traits are common in the plant kingdom but they usually only concern two distant genera and not three closely related ones. Based on this assumption, the nearest original ancestor of the *Lentibulariaceae* would have been a rosetted plant with no central root system and only one type of gland on the surface of its leaves (the digestive gland common to all three modern genera). All three genera generate rosetted plants (admittedly less obvious in the more recent *Utricularia* species). Their central root system never develops out of the seed coat during germination so that one end of the central stem grows while the other one dies off (only adventitious roots, *i.e.*, side roots developing from the main stem, are generated by *Pinguicula* species). With just digestive glands, it is hard to foresee how this original ancestor could have caught any prey since Lentibulariaceae digestive glands do not secrete mucilage and they lay flat on the leaf surface. Moreover, this type of gland secretes and absorbs via cracks in its cuticular surface (free exchange of substances between the outside environment and the inter-cellular apoplastic fluids) so that these mechanisms can only take place in an aqueous environment and death of the plant by dehydration can quickly occur if the glands are exposed to open and dry air. This need for an aqueous, or at least very humid, environment next to the digestive glands is still valid for all three modern genera. Therefore, it is very probable that the original ancestor was acting like a partially saprophytic-type plant (still carnivorous) in or next to a rich liquid broth. Most likely, the amount of water surrounding the leaves was small, like a thin film above the leaves or leaves wetted very often by rain or fog, to prevent the secreted enzymes from being diluted away. In such an environment, the secretion of enzymes would have helped degrade the surrounding organic debris to further enrich the broth next to the leaves. The attachment of the glands to tracheid element would have facilitated the distribution of these nutrients through the plant. So, the leaves (via the digestive glands) would have done the roots' job in feeding the plant. This may explain why an elaborate root system may have disappeared.

Hypothesising that carnivory in the Lentibulariaceae could have evolved from the above mentioned nutritional specialisations is new and different from previous hypotheses elaborated for other carnivorous plant groups. It has been suggested that carnivorous structures in Droseraceae (Schlauer, 1997) evolved from salt-secretion glands and/or as a flower defence mechanism aimed at gluing crawling insects to prevent them from pollinating or eating flower parts. Possibly, this explains why *Drosera* glands both secrete mucilage and digestive enzymes in contrast to *Pinguicula* which have two separate gland types to conduct these two activities.

The geographical distribution of the common ancestor of the Lentibulariaceae is impossible to guess with the current data. Except for their most derived members, *Pinguicula* is a northern hemisphere genus while *Utricularia* and *Genlisea* are southern hemisphere genera (see below). Possible explanations include that the ancestor of Lentibulariaceae was present before the split of Gondwana and Laurasia, or that in some stage of the evolution of the lineage diasporas were dispersed into both hemispheres to give rise to all 3 genera. Rather rapid distribution must have also occurred in the aquatic members of *Utricularia* to and throughout the northern hemisphere. This, however, seems rather likely given that many aquatics posses cosmopolitan ranges and are easily distributed by waterfowl.

For some reason, the common ancestor of the Lentibulariaceae did not survive and its descendants developed two separate strategies to better retain their catches and limit dehydration of their leaves (Müller *et al.*, 2002). One involved the production of a novel type of gland, a pedunculate gland, by the current *Pinguicula*. This has lead to a slowing-down of the rate of DNA evolution of its members. This genus-specific gland serves many new functions (reviewed in Legendre, 2000). It allows good retention of the prey via the production of mucilage and the creation of a bowl (leaf curling) under the prey. This movement is achieved thanks to the basal, epidermal cell that is swollen (water reservoir) in the resting state but secretes all of its content upon mechanical stimulation by a prey. In doing so, it looses turgor and sinks within the epidermis, creating a small depression under the prey where digestive fluids can be kept. This novel type of gland also prevents dehydration of the leaf thanks to the mucilage that surmounts it.

The different phenotypes of *Utricularia* and *Genlisea* apparently evolved via a separate option that consisted of closing the leaf on itself and forming inverted traps. Several species of *Pinguicula* have been observed to accidentally generate Champagne cup-shaped leaves under cultivation. This phenomenon rarely repeats itself on the same plant and seems to be a one-time error in the developmental program of the leaves. Close examination of a herbarium specimen of *P. utricularioides*, a *Pinguicula* species that naturally forms bladders, did not reveal any clue to the way these bladders evolved or whether they are a constant phenomenon for these plants. It is possible that some evolutionary intermediates of the bladder plants harboured Champagne cup-shaped leaves on a regular basis. *Pinguicula*-derived Champagne cup leaves are small and so are most of the bladders of *Utricularia* and *Genlisea*. Thus, *Utricularia* and *Genlisea* traps would technically be leaves in agreement with Lloyd (1942). Even though this leaf configuration offers an intermediate stage towards the development of a proper bladder, it does not allow the catching of many preys, nor their complete retention. Inverting tropism is one way to solve this issue and this is what *Utricularia*'s and *Genlisea*'s common ancestor may have done. Once the traps grow downwards, the flow of sap through their petiole will move backwards, pulled by dehydration of the top portion of the plant. Because of this inverted sap flow, the trap will now act as a permanent suction device that will filter its bathing medium. It will also be able to sit in a moist environment while allowing the rest of the plant to develop above the soil and/or water. The catches will be more numerous and can be anything from dissolved nutrients to microscopic organisms that will be caught and digested to be later assimilated. This scheme is further supported by a recent observation that *Genlisea* feeds on protozoans (Barthlott *et al.*, 1998) and the fact that some of the most ancient *Utricularia* species (members of section *Pleiochasia* like *U. volubilis* and *U. helix*) produce traps on the top of green petioles and that these petioles grow in several directions from upwards, to horizontal, to downwards. At present, no plants with inverted Champagne cup-shaped

leaves are known. Probably, they have not survived the competition with their more modern descendants. One possible reason for this is that such a trap is too open and can be visited by large creatures. During such visits, previously trapped microscopic organisms can be stolen and the trap or the head cells of the glands damaged. The head cells occupy a vulnerable position on top of a fragile stem. Because the traps are thought to exist in water, or at least in a very moist environment, infections would spread easily from such wounds. It is suggested that the closing of the leaf was the safest option and the best way to retain catches (formation of trap). *Genlisea* and *Utricularia* have developed two separate trap designs (Müller *et al.*, 2002a).

Genlisea traps produce two long arms to allow the pumping of water from a larger surface area. Trichome hairs are produced inside the trap. They are oriented towards the inside of the trap to prevent large preys from escaping. Attachment hairs are also generated to attach the two edges of the arms, while still leaving entry holes in between them. The role of the mucilage glands on the outside surface of the traps is unknown but Lloyd (1942) has been hypothesised that they generate a lubricant to facilitate the flow of prey towards the digestive cavity where the main digestive glands are found.

Instead of developing parallel series of hairs to retain prey, *Utricularia* generate a door and a doorstep to shut the trap. Interestingly, *Genlisea* species possess a non-functional door and doorstep on their dorsal and ventral sides respectively, close to the mouth of their traps. The proper functioning of a door system requires the presence of side features such as mucilage glands to seal the door (morphologically different from those of *Genlisea*), nectar glands to attract prey towards a tiny trap, and trigger hairs to allow future meals to induce the opening of the door (morphologically different from those of *Genlisea*). If our original hypothesis that *Genlisea* and *Utricularia* traps are in fact leaves that replace the function of the missing roots, how can *Utricularia* plants survive with tightly closed traps? The answer probably lies with the last type of gland that we haven't discussed so far. It is a tiny gland situated on the outside surface of the *Utricularia* trap that resembles the mucilage gland of *Genlisea* or the bifid/quadrifid glands of the inside of the *Utricularia* trap, except that it harbours only one small head cell. These glands have been observed to absorb nutrients readily (Lloyd, 1942). Their smaller size may make them less subject to mechanical damage by passing macro-organisms.

Even though complex in structure, the *Utricularia* trap has obviously been successful judging by the diversity of habitats in which these plants grow, the number of species in this genus and their geographical distribution. Their success has been suggested to have more than one origin (Müller *et al.*, 2002a). First of all, the transformation of leaves into underground structures that may compensate for the lack of roots has led to some unique changes in the Biology of other organs. Lloyd (1942) proposed that the green leaf-like organs could in fact be flattened stems. This proposal was later rejected by Taylor (1989) without specific reasons. Lloyd's conclusion was based on the study of organ development in newly developing seedlings. For many *Utricularia* species, he observed that the tiny seeds contain small food stores, develop small cotyledons and then form traps before green shoots develop from their base like a side bud at the base of a leaf petiole. The function of this early trap is most likely to provide food for the seedling in the absence of stores and photosynthesis. This may explain why the germination of *Utricularia* seeds in collections is so difficult since most of the germination media are made fresh and are, therefore, devoid of established microflora on which the young seedling (invisible above the ground at that time) could feed. The proposal by Lloyd (1942) is supported by the fact that many *Utricularia* species generate traps (*i.e.*, leaves) along the veins of their green organs (*i.e.*, lateral stems). Some of these lateral stems sometimes serve parallel functions such as underground storage organs (tubers, or tuber-like just like potatoes) or stolons along which traps (*i.e.*, leaves) can develop. At the base of these traps new lateral green shoots can be formed that generate a new rosette. It is clear from our genetic analysis that these side shoots have become more diverse through evolution to prevent concentrating all of the traps near the base of the main rosette as in the early forms of *Utricularia* (section *Polypompholyx* and *Pleiochasia*) and in *Genlisea*. Not all of the green organs have to be stems. As stated earlier, Some *Utricularia* species of section *Pleiochasia* harbour

traps at the end of green petioles. Thus, for some species there is a possibility that the green organs are petioles that do not bear any trap and express positive tropism. The unrelated carnivorous genus *Nepenthes* also produces leaves with well-formed traps and small green laminae upon germination. Later, when *Nepenthes* plants reach abundant food supplies, they may only form green leaf petioles without the expense of producing traps on their tips (Juniper *et al.*, 1989). A similar situation occurs in a third unrelated carnivorous genus *Triphyophyllum* (Bringmann *et al.*, 1999). Carnivory may, therefore, just be a more efficient way than photosynthesis to obtain food for a fast developing seedling. After all, meat is more calorific than vegetables (Juniper *et al.*, 1989).

Part of the success of *Utricularia* and *Genlisea* stems from their high DNA mutation rates. Such increased mutation rates (highest of the plant kingdom) may be due to the greater reliance of these species on carnivory (Müller *et al.*, 2002b). These plants may be considered to be the only obligate carnivores of the plant kingdom because they permanently suck nutrients and preys (at least microscopic ones) from their liquid environment unlike the other carnivorous plants which may be considered as occasional carnivores because they rely on an unpredictable source of prey. Additionally, and as stated above, the unique biology of these plants forces them to rely purely on their carnivorous nature at the seedling stage. Consequently, these plants have a lower dependence on basic metabolic processes such as photosynthesis, energy production, or amino acid and nucleotide synthesis. This, in turn, relaxes the selection pressure for maintaining the same composition of the DNA molecule that codes for these metabolic processes. Even though only metabolic genes should be affected by this increased evolutionary rate, recent findings (Müller *et al.*, 2002b) suggest that increased mutation rates affect a larger part of the genomes of these plants. This gives them a unique opportunity to generate new species faster for varying needs or to occupy new ecological niches. Because of the large genetic differences that quickly arise among species, it is not surprising that *Utricularia* and *Genlisea* are genera where no interspecific hybrids have been reported (even between closely related species that live together). This lack of back crossing to wild-type populations probably adds a snowball effect for increased genetic differences among species.

The shorter-term history of *Pinguicula*, *Utricularia* and *Genlisea* is much more difficult to reconstruct from the available data. For *Pinguicula*, our current sampling is admittedly too weak to provide a full picture of this genus. Nevertheless, it seems likely that *Utricularia* and *Genlisea* originate from the southern hemisphere since only their most recent members are in the northern hemisphere. Only some of these recent members can withstand a long period of frost. A contrary picture can be drawn for the set of *Pinguicula* sampled for this study. This set of *Pinguicula* species would have originated from Eurasia (between the Alp and the Koreas) because *P. alpina* is the modern descendant of the ancestor of the *Pinguicula* species of the Americas and West Indes. The genus entered North America via the Bering sea or via Greenland. Upon arrival in Mexico and/or southern USA, the winter hibernaculum would have been transformed into a drought-resistant *Sempervivum*-type rosette (only a recent group of species never forms any winter-resting structure; Ex: *P. filifolia*). This change may seem big but is not because frost-resistance and drought-resistance are achieved by the fixation of water molecules to prevent them from forming ice crystals or evaporating away. During the alternating periods of cold and warm times, it is probable that *Pinguicula* plants have repeatedly moved down into valleys where they could merge and hybridise to then isolate themselves (and differentiate from each other via independent evolution) by following the glaciers north or towards the top of mountains. Most of the European *Pinguicula* species (plus *P. vulgaris* and *P. macroceras* in North America and Asia) would thus have been generated because of the geographic isolation of their populations after the last glaciation ages. Because this event is very recent, it may explain why we could not find any genetic differences among most members (*P. leptoceras* and *P. poldinii* excluded). This theory supports the hypothesis of Steiger (1998) that these species evolved from southern to northern Europe by increasing their ploidy. Casper (1966) has, however, suggested a very different scheme of evolution for *Pinguicula* species. He proposed that the genus originated during the Miocene on

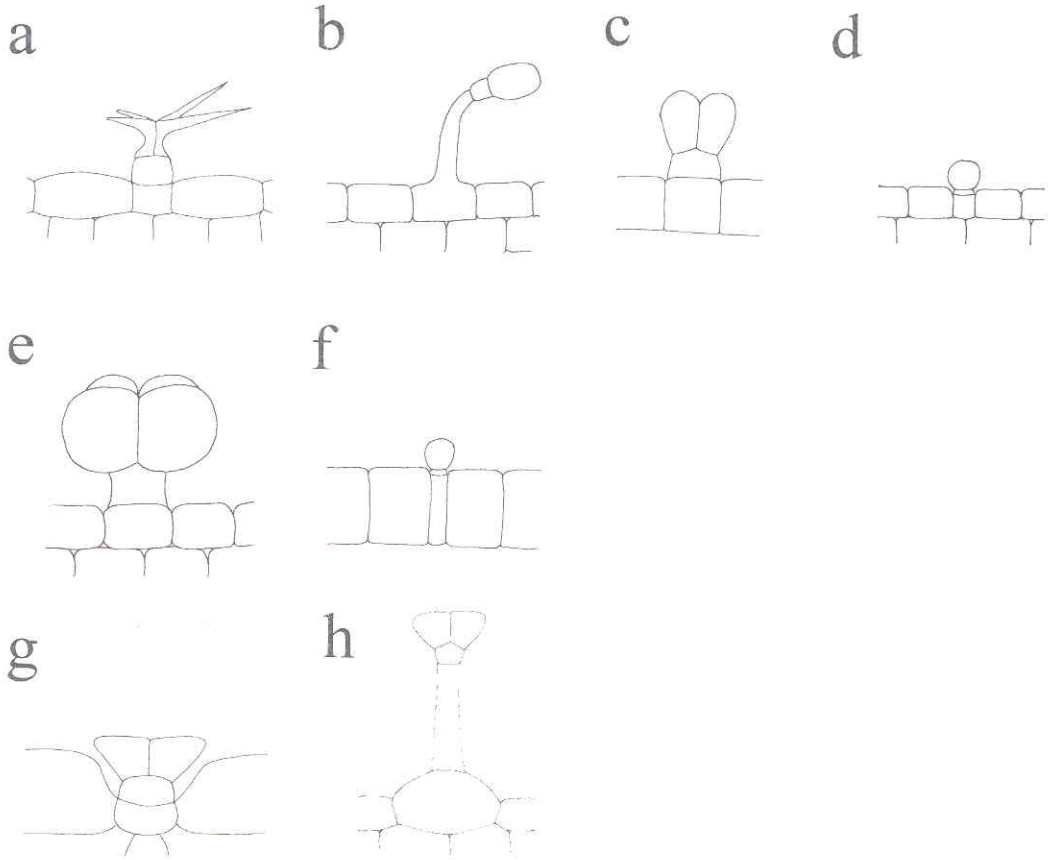

Figure 1. Anatomy of the glands of the Lentibulariaceae. (a) Digestive gland (quadrifid) of *Utricularia*. Some digestive glands possess only 2 head cells (bifid glands); (b) Mucilage glands of *Utricularia*; (c) nectar gland of *Utricularia*; (d) external gland of *Utricularia* trap; (e) digestive gland of *Genlisea*; (f) mucilage gland of *Genlisea*; (g) digestive gland of *Pinguicula*. They may possess 4 to 8 head cells; (h) mucilage gland of *Pinguicula*. They usually possess 8-32 head cells. Reproduced from Lloyd (1942) and Juniper *et al.* (1989).

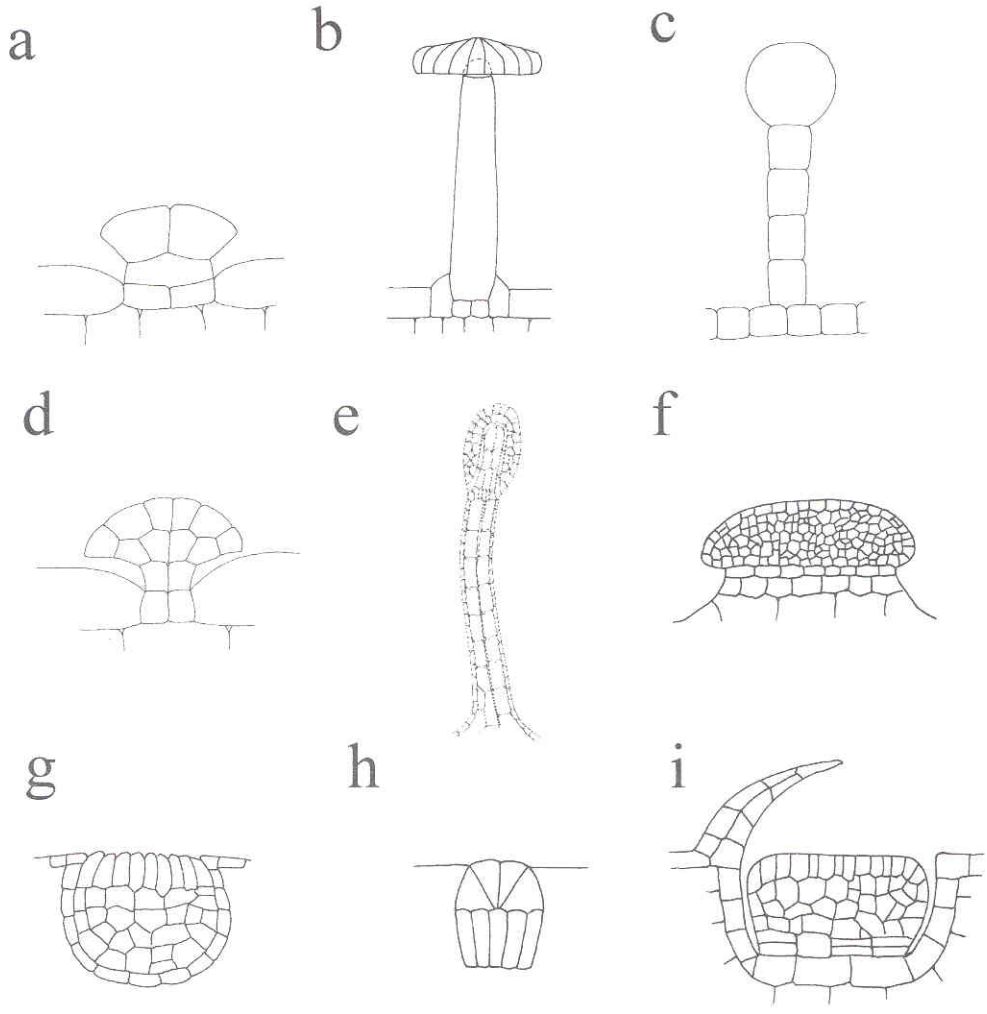

Figure 2. Anatomy of the glands of carnivorous genera not belonging to the *Lentibulariaceae*. (a) digestive gland of *Byblis*; (b) Mucilage gland of *Byblis*; (c) digestive gland of *Ibicella*; (d) digestive gland of *Dionaea*; (e) digestive gland of *Drosera*; (f) digestive gland of *Triphyophyllum*; (g) digestive gland of *Cephalotus*; (h) digestive gland of *Sarracenia*; (i) digestive gland of *Nepenthes*. Reproduced from Lloyd (1942) and Juniper *et al.* (1989).

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The Structural Basis for Nutrient Transport in the Pitchers of *Nepenthes*

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The large multicellular digestive glands in the lower portion of the pitchers of *Nepenthes alata* are active in transport of materials into and out of the trap. Moreover, the function of the glands appears to be developmentally regulated. Underlying vascular tissue is closely associated with the glands and provides a mechanism for fluid delivery into immature pitchers while they are still closed. This proximal placement also provides an efficient mechanism for transport from the digestive fluid in mature pitchers to the rest of the plant. Ultrastructural localization of the heavy metal tracer lanthanum shows mature glands, similarly to other carnivorous plants, have thick endodermal-like waxy deposits at the gland base to prevent unregulated movement of materials through the cell wall space. Fluorescent tracer studies show mature pitchers can transport fluid from the pitcher into the underlying vascular tissue while transport in the reverse direction becomes blocked when the trap matures.

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Comparative physiology of the Droseraceae *sensu stricto*— How do tentacles bend and traps close?

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Abstract: The family Droseraceae, which currently includes only *Drosera*, *Dionaea* and *Aldrovanda*, is related to *Drosophyllum* and *Nepenthes*, and also to the Plumbaginaceae and Polygonaceae. *Plumbago* has *Drosophyllum*-like hairs on its sepals which catch insects and secret digestive enzymes upon stimulation. All members of the Droseraceae *s.s.* have active traps, which respond rapidly to mechanical stimuli with capture movements that are mediated by action potentials triggered by mechanical stimuli. In all of these plants the rapid bending and closure movements involve rapid acid growth but in *Aldrovanda* a turgor loss mechanism may play an important role as well. A slower set of secondary movements initiated and maintained by both mechanical and chemical stimuli act on closing the whole leaf in *Drosera*. In all members of the family these slower movements, which are mediated by auxin at least in *Drosera*, maintain the closure until the prey is completely digested and ceases to release chemical signals. The question of rapid acid growth in *Dionaea* has caused some controversy because most very rapid action potential mediated plant movements are due to turgor loss mechanisms that involve the loss of K^+. However, experiments with buffers in *Dionaea* indicate that acid growth is the most important cause of movements in the *Dionaea* trap. Neutral buffers can prevent movements in *Dionaea* and still allow the action potentials to occur in response to mechanical stimuli. *Aldrovanda*, *Dionaea* and *Drosera* also have a loss of ions from the cells during the action potential. This may assist in rapid early phase closure in *Dionaea* and may be the main force in early phase closing of *Aldrovanda*. All cells in both *Dionaea* and *Aldrovanda* traps have been shown to be excitable. It appears to be the anatomy of the sensory organ and the trap lobes that determines which cells have a sensory function and what the pathway of the action potential is rather than the membrane properties of the cells. In addition the anatomy of the trap lobes accounts for much of the rapid movement by allowing them to flip shut in a way that greatly amplifies cell size changes in the lobes of *Dionaea* and *Aldrovanda*.

It is probable that the original Droseraceous carnivorous plant was a sticky haired stationary trap, such as in *Drosophyllum*. These traps probably originally served another function such as defense against insect predation or pollen theft. Cladistic analysis of DNA from both the matK gene (Meimberg *et al.*, 2000) and the rbcL gene (Albert *et al.*, 1992; Williams *et al.*, 1994; Fay *et al.*, 1997) has revealed that the members of the Droseraceae are closely related to an as yet unnamed natural sister group of plants that includes the Nepenthaceae, Dioncophyllaceae, Ancistrocladaceae, and *Drosophyllum* (now Droserophyllaceae but formerly considered to be in the Droseraceae). Other closely related families include the Polygonaceae and Plumbaginaceae. In the Plumbaginaceae *Plumbago* has *Drosophyllum*-like glandular hairs on its sepals that capture prey and are capable of being induced to secrete proteolytic enzymes (Stoltzfus *et al.*, 2002). The pitfall trap of *Nepenthes* and the snap-trap of *Dionaea*/*Aldrovanda* are probably independent modifications of a trapping system that began as an adhesive trap similar to that of *Drosophyllum*. None of the plants in the sister group has rapid movements involved in prey capture but active traps characterize all of the Droseraceae *sensu stricto* (*Drosera*, *Dionaea* and *Aldrovanda*). It is the movements of the Droseraceae that will be compared here.

Drosera traps look deceptively simple. Although the trap of *Drosera* is often referred to as passive, Lloyd (1942) correctly characterized it as an active trap. Its movements, which bring a fly captured on the marginal tentacles to the center of the leaf and then envelop the prey and digest it, resemble those of a very slow hydra (Williams, 1976). The individual tentacles respond to direct stimuli by producing action potentials that terminate near the base of the tentacle (Williams and Pickard, 1972; Williams and Pickard, 1980). Individual tentacles respond to the action potential with a rapid growth movement directed toward the center of the leaf that carries the prey in that direction. The stimuli that cause these responses are mechanical but are greatly enhanced by chemicals such as Na^+ and NH_4^+ (Williams and Pickard, 1980; Suda *et al.*, 2002). These responses begin 15 seconds after an action potential and are completed in a few minutes. The bending movement is due to differential growth since the outer cells of the tentacle expand more than the inner ones during bending (Hooker, 1916, 1917). A slower response begins in as soon as a few minutes and can take from an hour to several hours to complete (Williams and Pickard, 1980; Suda *et al.*, 2002). This secondary bending response involves the bending of tentacles not directly stimulated by the prey and also the blade of the leaf itself and requires that both chemical and mechanical stimuli be present. It is mediated by auxin as many growth movements are (Bopp and Weber, 1981). Here again the chemical stimuli include Na^+ and NH_4^+. Secretion of mucilage and digestive enzymes is also stimulated and the captured prey is digested. Unbending seems to occur when chemical and mechanical stimuli are no longer present. The slow unbending movements are also due to differential growth since the inner cells expand relatively more during unbending (Hooker, 1916, 1917).

Dionaea is closely related to *Drosera* but has a trapping mechanism that superficially appears to be very different. In reality it is a modification of the *Drosera* trap. The physiology of the *Dionaea* trap resembles the *Drosera* trap in many ways. Both have rapid action potential mediated movements that begin in a sensory hair and spread. Unlike that of *Drosera*, the action potential of *Dionaea* spreads throughout the trap and quickly triggers a rapid movement in the entire leaf blade. Chemical stimuli are not involved in this response. The mechanically stimulated signal begins in a trigger hair that is probably a highly modified tentacle (Williams, 1976). It even has an endodermis, a layer found in secretory structures of plants, such as the *Drosera* tentacle, although it does not secrete anything. If the trap has captured a visitor, chemical stimuli from the digested prey provide an additional stimulus which keeps the trap closed until the stimulus ceases (Lichtner and Williams, 1977). Just as in *Drosera*, the *Dionaea* trap shows a net increase in size during closure and during reopening. This is the plant physiologist's definition of growth. But the speed of closure conceals this mechanism. Rapid movements in plants that are triggered by action potentials are often caused by rapid loss of turgor in "motor cells" and growth is traditionally thought of as a slow process. Hooker (1916, 1917) called the relatively slow movement of the *Drosera* tentacle a growth movement. Brown (1916), on the basis of evidence identical to Hooker's, called *Dionaea*'s movements a turgor movement. Turgor movements are often fast and triggered by action potentials. Growth movements were not known to be fast or triggered by action potentials. The description of *Drosera*'s movements as growth movements and *Dionaea*'s movements as turgor movements meets expectations but does not explain the experimental data.

Is *Dionaea* closure a turgor movement or a growth movement? Solute and turgor pressure changes are involved in both growth and turgor movements. The difference is in what happens in the cell walls. In turgor movements the cell walls remain elastic acting as a girdle that presses inward on the cells. During growth the wall's fibers loosen and the turgor pressure within the cells then causes them to expand. Finally solutes taken up by the cells restore the osmotic pressure and thus restore the turgor pressure. In slow growth these two processes overlap and a relatively smooth expansion occurs.

Turgor movements are caused by the gain or loss of water from cells that is driven by the gain or loss of ions or sugars from the cells. All known rapid turgor movements, such

as those in *Mimosa*, are caused by a turgor loss due to a loss of K^+ with associated anions and the subsequent loss of water from the cell (Satter, 1979).

Acid Growth, a concept first used in the 1970s to describe the action of auxin, now has a much more general explanation. Plant cells grow because cell wall proteins known as expansins loosen the wall by allowing slippage between the hemicelluloses that form the matrix that binds cellulose microfibrils together. This loosens the wall fibers and lowers the inward force the wall exerts on the cell. The turgor pressure of the cell then causes it to expand. (Turgor pressure drives both growth movements and turgor movements.) Expansins have an acid pH optimum. Decreasing the pH of a cell increases wall loosening. Auxin stimulates growth by stimulating the pumping of H^+ into the cell wall compartment from the cytoplasm. Any process that causes the cell wall compartment to become acid is capable of stimulating acid growth in any growing plant cell (Cosgrove, 2000). Addition of expansins to cell walls results in expansion of cell walls within seconds (Cosgrove, 2001). The slippage caused by expansins performs the role that cleavage of hemicelluloses by hydrolytic enzymes in the cell wall was once though to have.

***Dionaea* Trap Closure** is the result of a flipping of the trap lobes from a position where the exterior of the trap is concave to one where the exterior is convex. This movement can begin as soon as 0.4 sec after stimulation and be completed in a second (Sibaoka, 1980). A slower movement follows which narrows the trap opening, ultimately resulting in the tight appression of the lobes. Both processes result in a net increase in trap cell volume (Fagerberg and Allain, 1991) but the cellular changes during the first fractions of a second have not been adequately measured. However, a greater increase in the volume of the outer layers of the trap lobes relative to the inner lobes supports the acid growth model of trap closure* as does a range of other evidence:

- Trap lobes show an irreversible increase in volume during closure (Fagerberg and Allain, 1991).
- Traps with walls perfused with neutral buffers are paralyzed but will still produce action potentials when the trigger hair is stimulated (Williams and Bennett, 1982).
- Traps perfused with lower concentrations of neutral buffers can be closed by multiple stimuli (Williams and Bennett, 1982).
- Traps with walls perfused with acid buffers will close without stimulation (Williams and Bennett, 1982; Hodick and Sievers, 1988).
- Significant ATP is used during trap closure. It is needed to move H^+ from the cytoplasm to the walls (Williams and Bennett, 1982).
- The surface of the outer lobes of the trap is more flaccid just after trap closure.
- Plasticity in the outer layers of the trap lobes increases dramatically during closure while that of the inner lobes remains unchanged (Hodick and Sievers, 1989).
- Ca^{2+} decreases in the cell wall and increases in the cytoplasm during the action potential (Hodick and Sievers, 1988). This decreases pectin cross-linking in the wall and increases the production of wall material in the cell.

The ability of neutral buffers to prevent trap closure without affecting the stimulation of action potentials in the trap is strong evidence that all phases of closure of *Dionaea* traps are caused by acid growth (Williams and Bennett, 1982). The three-fold increase in electrogenic H^+ pumping that is estimated to occur during the *Chara* action potential (Thiel, et al., 1997) demonstrates the feasibility of the same process in *Dionaea* traps. By contrast, Hodick and Sievers (1989) have criticized the acid growth model of trap closure based on the their determination that the amplitude of extracellularly measured action potentials was the same before and after closure in traps while intracellularly recorded action potentials from traps perfused with acid buffer show a decreased amplitude. The comparison of extracellular measurements to intracellular ones is problematic as is the comparison of cells

* Brown (1916) and Williams and Bennett (1982) assumed that the epidermis was responsible for trap movement. Fagerberg and Allain (1991) have demonstrated that the expansion takes place primarily in outer mesophyll cells. The acid growth model is supported by the new and more accurate data.

exposed to acid conditions for periods of minutes in one solution to those exposed for seconds or fractions of seconds in another. The conclusion that cell wall acidification does not occur based on this data is not strong evidence. However, Hodick and Sievers (1989) also demonstrated a differential plasticity increase in the outer cell walls of the trap lobes relative to the inner ones during trap closure. This is strong evidence in favor of a growth model of trap closure. The decrease in turgor of the outer walls, expected if the trap expanded by rapid wall loosening, also supports this model (Williams and Bennett, 1982).

There is almost certainly a loss of turgor due to a loss of ions during the action potentials during the earliest phase of trap closure. Freshwater and land plants have relatively low ion concentrations in their cell walls. If *Dionaea* and *Aldrovanda* work by the same mechanism as giant Characean algal cells (Thiel, et al., 1997), and all evidence that exists indicates that they do, the cells would loose both Cl^- and K^+ during the action potential. This would result in a loss of turgor during these ion fluxes. The movement of Ca^{2+} into cells would have the opposite effect but a smaller one since its flux is lower and since Ca^{2+} carries twice the charge per ion. Evidence in favor of involvement of turgor movements during early closure is:

- K^+ and probably Cl^- are lost during the action potential—these would result in a loss of turgor (Iijima and Sibaoka, 1983, 1984; Hodick and Sievers, 1988).
- Many rapid plant movements are turgor loss mechanisms.

There is certainly turgor loss resulting from the loss of ions during the closure of the *Dionaea* trap but it is unclear how much of a role it plays in causing the rapid movement. The fact that traps can be paralyzed by neutral buffers that do not prevent action potentials indicates that its effect is minimal in *Dionaea*.

Aldrovanda is a third genus in the family that resembles *Dionaea*. Its trap is a smaller faster aquatic version of the *Dionaea* snap trap. *Aldrovanda* had a great deal of excellent work done on it in the 1980s by Iijima and Sibaoka. The results parallel those of *Dionaea* very closely with some important differences:

- A single stimulus will close a trap but two are required in *Dionaea* (Iijima and Sibaoka, 1981).
- Trap closure is about ten times faster than in *Dionaea* (Iijima and Sibaoka, 1981).
- The trigger hair bends at what appears to be the vestigial endodermis rather than below it (Iijima and Sibaoka, 1982).
- There are only three cell layers. The epidermis must play a larger role in trap movement because it is a larger part of the trap (Iijima and Sibaoka, 1982).

Iijima and Sibaoka (1983, 1984) first provided the information on ion fluxes during the *Aldrovanda* action potential. Similar experiments on *Dionaea* were performed later by Hodick and Sievers (1988) with nearly identical results. The following results were found in *Aldrovanda* and *Dionaea* studies:

- All cells in the *Aldrovanda* and *Dionaea* traps are excitable (Iijima and Sibaoka, 1981; Hodick and Sievers, 1988).
- The action potentials in all cells of both *Aldrovanda* and *Dionaea* traps are similar (Iijima and Sibaoka, 1981; Hodick and Sievers, 1988).
- There is a low resistance between cells of the trap of *Aldrovanda* through which the action potential spreads (Iijima and Sibaoka, 1982).
- The loss of ions in *Aldrovanda* may contribute significantly to the early rapid flip of the trap (Iijima and Sibaoka, 1983).

Some general conclusions can be drawn from a comparison of *Aldrovanda* and *Dionaea*.

- Since all cells of the trap are excitable the sensory cells in the trigger hair are probably not physiologically unique. They are simply placed in a situation where they can be mechanically stimulated. Their uniqueness is more anatomical than physiological.
- Since all cells of the trap are excitable the pathway of the action potential and therefore the responsive area of the trap is determined not by which cells are excitable but by which

cells are excited by electrical current from their neighbors. Again the anatomy seems to determine the function.

The distribution of plasmodesmata in trap cells deserves further study and the anatomy of these traps in general needs more attention to detail but we have an emerging picture of how they work. The uniformity of trap cell function contrasts with the diversity of structures of trap components. It is likely that the rather complex picture of cell expansions and contractions seen by Fagerberg and Allain (1991) are more due to the pressures that the two lobes and the various cell layers place on each other than to differences in membrane physiology in the various cells. Families of expansins exist and the genes for them are turned on only in specific tissues. This is a way plant cell growth is controlled. It is also probably how Venus flytraps close.

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Changes in trap tissue relationships during closure/reopening in Venus's flytrap (*Dionaea muscipula* Ellis): A possible model to explain trap morphological changes

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Introduction

It is obvious that plants exploit animals almost as much as animals exploit plants. However, it is seemingly rare that this exploitation takes the form of capturing and digesting other organisms. When it does it peaks the interest of both scientists and non-scientists. I would guess that trap movement in Venus's Fly Trap *Dionaea muscipula* (Ellis) has fascinated people since the first person observed it happen. Early on scientist like Charles Darwin and his son 1875 began to study this phenomena to try and explain how plants, without muscles, effected such spectacular movements.

The movement associated with closure of the Venus's Fly Trap (VFT) trap that Darwin and many others studied is really a series of movements: 1) a rapid movement which occurs in less than a second, where prey is captured and escape made unlikely; and 2) a slow movement which extends for tens of minutes to an hour that ensures prey capture and forms the digestive pocket. Trap closure in VFT involves a complex series of leaf (trap lobe) movements initiated by stimulation of the adaxial trigger hairs (trichomes), or some form of mechanical /electrical/chemical irritation of trap lobe tissue (Darwin, 1875; Brown, 1916). The closure process is initiated after a membrane localized depolarization wave travels (at approximately 10 cm/s) through all five trap tissues in the stimulated trap lobe and across the midrib to the adjacent lobe (Sibaoka, 1969; Williams and Mozingo, 1971; Jacobson, 1974; Williams and Bennett, 1982; Hodick and Sievers, 1986; Hodick and Sievers, 1989) for review (Juniper *et al.*, 1989). During the one-two hours (approximate) required for complete closure of unfed traps (Fegerberg and Allain, 1991), considerable trap morphological change occurs. These morphological changes have been described in various detail by a number of researchers (Darwin, 1875; Brown, 1916; Lichtner and Williams, 1977; Williams and Bennett, 1982; Fagerberg and Allain, 1991) for review: (Juniper *et al.*, 1989). The motivating force for movement of the flytrap lobe has been described as a resulting from a change in the balance between tension in the epidermal and compression in the "pith" mesophyll tissues. This shift is presumably created by unequal growth forces within the these tissues (for review, Juniper *et al.*, 1989). Hodick and Sievers (1989) concluded that mesophyll tissue provide the driving forces for trap movement while epidermal cell extensively controls the expression of that movement. The importance of epidermal tissue flexibility in controlling growth patterns (direction) and localization of growing force in central "pith" tissues has been proposed for a number of plant morphogenic events (Kutschera, 1989 and references therein).

Early models of trap movement proposed that growth of outer (adaxial) cells closed the trap and growth of inner cells (abaxial) open the trap (Brown, 1916). Brown's study involved placing ink spots on the trap lobe at known distances then measuring the changes in distance between the marks as the trap closed and reopened. Unfortunately, there were no statistically significance differences between the marks before closure and

after reopening which made the results difficult to clearly interpret. Added to this was the fact that the morphological changes the lobes undergoes during closure make such measurements difficult under the best of circumstances.

In our lab we observed trap closure with time lapse video and from that study described distinct stages of closure and reopening defined by specific morphological changes in the trap. Based on the information gained from the videos we had a difficult time in trying to reconcile previous models of closure. In order to explain the changes we observed on the videos we (Fagerberg and Allain, 1991) felt that changes in trap morphology must be brought about as a result of a progressive sequence of specific, localized, tissue expansion events which occurred during defined post-stimulation time periods. In subsequent studies we developed information that indicated that activity in all five of the tissues which comprise the trap lobe was essential to explain lobe morphological changes. Also, that opposing tissue groups (abaxial and adaxial) were simultaneously involved in trap closure and re-opening events. Since the trap tissues are anatomically distinct from one another and are characterized by a complexity of cell to cell adhesion sites we felt they could easily act independently of each other (Fegerberg and Allain, 1991; Fagerberg and Howe, 1996).

In order to alter trap morphology of cell flexibility within the trap must be altered in some way. This may occur either through cell growth or changes in nature of the cell wall. Such changes have been associated with an efflux of H+ from cells. However, there is some disagreement as to the exact role of protons in eliciting these changes (Ashida, 1934; Sibaoka, 1969; Williams and Pickard, 1980; Williams and Bennett, 1982; Williams and Bennett, 1983; Hodick and Sievers, 1989). The question of the ability of water flux between the cells and its surrounding to cause such rapid movements (10s of milliseconds to 1s-Morillon, Lienard *et al.*, 2001) has also been raised. Perhaps a mechanism similar to muscle contraction and contractile proteins that modify cell shape (*e.g.* ratio of length to diameter) without the need to change the volume (Morillon *et al.*, 2001). There is still work to do to answer these questions.

OUR MODEL: Our data indicated that specific cells in one region of the trap underwent changes in size during specific times, while other cells of the same tissue in other regions of the trap remained unchanged. Differing from previous model we also noted that cells on opposite sites of the traps were involve in these events. We speculated that the degree and direction of lobe bending in these traps is controlled by the relative force generated by opposing tissue groups (tissues on abaxial/adaxial sides of the medulla, Fegerberg and Allain, 1991). Contact of opposing trap lobes during the second stage of closure (Appression stage, Fegerberg and Allain, 1991) would establish a pressure gradient which may have equalized forces and aided in coordinating movement between the two lobes during closure. If these observation are borne out then trap closure and reopening is very complex movement. To our knowledge no movement in plants has been postulated or described with this level of complexity.

The dynamics of trap reopening have not been studied to the same extent as trap closure, yet the morphological changes are as great, albeit slower. The same five trap tissues involved in trap closure must somehow work in concert to create a morphological effect opposite to that of closure. Observations from time-lapse video images of trap reopening showed that the sealed, recurved, closed trap morphology was returned to that of an open, pre-stimulated trap through a series of coordinated flattering movements. These movements were a reversal of those responsible for closing the trap. Whether the tissue dynamics associated with closer are reversed during opening has been addressed and it appear that they are not similar (Fagerberg and Howe, 1996). The major questions remain as to how and where reopening forces are generated in the trap? In this talk we address aspects of that question namely, the changes in relationships of tarp tissues to each other as reopening occurs. This presentation will provide the data upon which we developed our model for trap movements.

We might also consider that the relationship between the mechanism of growth that

closes/opens the trap and other types of plant growth such as nastic movements, tropisms, flower opening etc. has not been established. It may be that there are many common mechanistic and control features between trap movement in VFT and other higher plant movements. Logic might dictate that, based on past experiences, we should not expect that VFT would have evolved a wholly unique method of plant movement completely separate from that of other types of higher plants. Therefore some or all aspects of trap closure and reopening may serve as a model system for studying other types of stimulus induced plant movement with the distinct advantage of the predictability of the movement and the rapidity with which it is accomplished. At the very least this is very interesting phenomena.

Perhaps because of its size and speed of movement VFT is one of the most spectacular of these plants, but as spectacular as it is it represents only one of a number of carnivorous habits. 1. ***Passive trappers*** like the pitcher plant. 2. ***Semiactive trappers*** like the sundew. 3. ***Active trappers*** like VFT, *Aldrovanda*, *Utricularia* etc.

My interest is in the mechanism for trap morphological changes but there is also a fascinating physiology here as well. I originally got interested in this problem while looking for a model system to study rapid cell expansion and its effect on organelle volume. I have since come to realize that VFT has the potential to be a model system for many types of plant behavior. I should mention that in our study only traps which had never gone through a closer event prior to our study were used. We found that subsequent closure events proceeded much more rapidly than those of first time closure traps and stages more difficulty to discern.

Defining the trap lobe – The trap is a modified leaf or part of leaf (?). It arises from the center rosette (corm) as a small notched bud. The edges of the notch begin to fold in as the bud begins to elongate and these in folded regions will eventually form the margins and tynes. The trap bud emerges from the center of the crown with the trap portion a narrow elongate structure folded towards the center of the plant. As the leaf emerges the trap portion unfolds and develops/enlarges and begins to open slightly. At this point the tynes, still folded into the center of the trap are visible. In the last stage of development the traps opens up, the tynes unfold and spread out and a functional trap is produced. There will be some enlargement growth after this stage.

This rather odd leaf development has some significance since the tynes and the trap margin that was previously folded into the center of the trap remains unable to generate a stimulation response (can not induce trap closure) – ***yet are the only part of the trap involved in rapid trap movements.*** Obviously the cells of this region have different developmental fate than those of the rest of the trap.

The pattern of marginal tyne placement is established by the location of secondary veins. Tyne development proceeds at the ends of secondary veins and where three tertiary merge into a single vein, this vein extends into the tyne. This whole region of anatomizing veins in the margin of the trap is not able to generate a stimulation response and differs anatomically from the rest of the trap.

Traps are connected to rest of the leaf by a neck region. Although anatomically very similar to the trap proper, the cells of the leaf blade do not move. However, one can stimulate trap closure by injuring these cells. The leaf proper has quite a variable morphology.

Once mature, the trap can be stimulated to close, normally by two consecutive stimulations of trigger hairs within seconds (it does not have to be the same trigger hair).

TRIGGER RESPONSE – Compression (or stretching) of cells in the sensitive zone (stretch receptors) of the trigger hairs (trichomes) set up a depolarization wave that spreads out from the triggers in a manner typical of depolarization waves in nerve cells. The efflux of H^+ from the cells and Ca^{++} into the cells initiates a charge flux which spreads to the next gated membrane channel and so on. The depolarization wave is passed from cell to cell possibly via plasmodesmata (Juniper *et al.*, 1898). Thus, the distribution and numbers of plasmodesmata may be very important in determining where and how much

signal passes to various cells. The depolarization wave propagates from stimulation source outward at about 10-40 cm/s. It must also cross the midrib and stimulate the second lobe. If time delay is too great, there is a mistake in closure trap lobes do not match and close unevenly. Thus, closure not only requires coordination within a lobe but also between two lobes.

Since there are at least two movements that result from trap stimulation (FAST and SLOW), there may be more than one mechanism at work to close the trap. Some cells may have a support rather then growth/movement role. For instance, if a cell pumps ions into the cell without cell wall "relaxation", then the cell becomes super turgid = rigid cell. This may serve as a sort of hydroskeleton or girder system to help support morphological change in the trap lobes. There is no direct evidence of this but the complexity of the cell dynamics I am going to describe at least leaves open the possibility.

From a functional point of view (not anatomically) the margins are very different from the rest of the trap. 1. They lack ability to initiation stimulus. 2. This zone is also the only region where truly rapid movement takes place, ***therefore it is sensitive to stimulation but cannot generate it.***

For the purpose of our study we divided the trap lobe into three regions: 1) A – which included the upper part of the lobe including the margin, 2) B- which was the middle portion of the trap lobe and 3) the lower portion of the trap lobe.

TRAP TISSUES – Within these lobe regions we have defined five trap tissues, two are epidermal tissues and three are mesophyll tissues. They are as follows: 1) lower (abaxial) epidermis, 2) lower (abaxial) cortex, 3) medullary (middle) tissue, 4) upper (adaxial) cortex, 5) upper (adaxial) epidermis. All tissues were characterized by intercellular spaces except for the epidermal tissues- this may be important in allowing trap lobe flexibility as discussed later. There are quantitative differences in the volume of the trap occupied by the different tissue in different trap regions. Also, the mesophyll cells of VFT, like most touch sensitive plants, have large tannin bodies.

Opposing tissues – These tissues were defined as paired tissues on either side of the medullary tissue, *e.g.* upper/lower cortex, or upper/lower epidermis. Within the cortical tissues there was considerable intercellular space, this was not true for the medullary tissue in all regions of the trap.

From our time lapse videos we defined the following stages of closure: If everything proceeds normally, we can recognize some major morphological changes (stages) associated with closure = A) CAPTURE < 1 second – in which the margin tissues were bent inward interlocking the tynes; B) APPRESSION $\leq$ 30 min where the lobes were pressed together, the margins in contact with each other; C) SEALED $\leq$ 1-2 hrs where the margins were tightly appressed forming a digestive pocket.

Trap closure: In order to effect the shape of the lobes during closure there has to be unequal growth on one side of the medulla or another, but it does not appear to be an all or none situation, *i.e.* tissue on both side of the medulla grow or get smaller in coordination with each other. The movement of the trap appears to be more like the operation of opposing muscle groups that control precise arm movements. If this is true, then we are looking at a very sophisticated plant.

In order to study tissue changes associated with trap movement we must fix, embed and section the trap thus, we can only measure a single trap once. Unfortunately, the same tissue cells in different traps are not the same size, therefore direct comparison between traps for different stages of closure are not really possible so we normalized our measurements. We measured cell lengths for each tissue type from each trap region, then we calculated the mean length for each cell type in each region. We summed up the means for each tissue in all three regions and then divided each regional mean by the sum = *Relative importance value* for each particular tissue in each region of the trap. Since Relative importance (RI) values are based on the relative size of a particular cell type compared against other similar cells in that trap we could use RI values to compare traps of different size. Each trap stage could be compared to next by comparing how these RI

relationships changed with the stage and by regions.

Anesthetization of traps – In order to stop trap movement at various stages of closure long enough to fix the tissue we anesthetized the traps. We did this by infiltrating them with EGTA in a pH 7.6 buffer. Data from stage of closure was gathered from three closure events and summarized for this presentation.

OPEN-CAPTURE – During the first stage of trap closure the trap changes shape from an open trap to the capture stage (0-5 min post stimulation). Based on RI values it appeared that the adxial (inner) tissues of the C region combined to push the bottom of the trap outward while the abaxial tissues of the B region and the central tissue of the A region bent the trap inward creating an arc with the margins facing each other. The very first part of this event, the margin inflexion (< 1 second) was to rapid to be studied using out techniques. Examination of this region before and after stimulation indicated a radical change in orientation of elongation of the marginal cells but this was hard to interpret accurately.

CAPTURE-APPRESSION = The arc of the trap is extended so the margins of opposing lobes come into contact (30-60 min post stimulation). We felt this change was brought about by growth of the middle tissues of region C, increases in abaxial and to a lesser extent adaxial epidermis cells of region B and increases in medullary tissue of region A.

APPRESSION TO SEALED = At this stage the margins are tightly appressed, to the point that the margin and tynes may recurve away from the center line of the trap. Rergion B and C are involved in forming a sealed digestive pocket (60 min + post stimulation). RI values during the transition from appressed to sealed stages indicated that the adaxial epidermal cells of region C increased slightly which would push the lower trap outward., the medullary cell of region B increased in size which, based on the curve of the trap served to force the margins more tightly together aided by increases in the abaxial cortical cells of region A.

TRAP REOPENING

Based on our time lapse videos of reopening the morphological changes during trap reopening appeared to be a reverse of those associated with closure. This being the case we wondered "were the tissue dynamics of reopening just a reversal of those that closed the trap"? We applied the same techniques used to study trap closure to study trap opening.

During reopening, we could recognize 3 major morphological stages. These were also defined from time-lapse video.

Stages: SEALED – (last stage of closure) – DEAPPRESSED – During this stage region A of the trap ballooned which opened the digestive pocket and release pressure of opposing lobes on each other. This was accomplished by increases in the adaxial epidermis of region C along with increases in the adaxial cortical tissues of region B. The adaxial and abaxial cortical and medullary tissue of region A also increase at this phase pushing the A region of the trap outward.

DEAPPRESSION- RELEASE. During this stage the trap margins separated and the trap lobes moved apart. This was accomplished by increases in the adaxial tissues of region C, larger increases in medullary tissues of region B and increases in both adaxial and abaxial cortical tissues of region A.

RELEASE-FULL OPEN. At this stage traps assumed almost the preclosure shape. Once a trap has been closed it never assumes the exact shape of the trap prior to closure. This change in trap shape was brought about by increases in the abaxial and adaxial cortical tissues of region C, increases in the abaxial epidermis of region B and increases in the adaxial epidermis of region A.

Support for activities of opposing tissues. Calculating correlation coefficients of changes in tissue activity we noted that highest values occurred with opposing tissues and

were positive in nature. This means that both outer and inner tissues changed in the same manner (i.e. either increased or decreased together). High correlation values between outer tissues and medulla also supports the possibility of its role in structural support against which the other tissue act.

Trap width changes – During trap closure there were no significant changes in trap width. However, during reopening – Region A showed significantly less trap width as reopening progressed. This may have been the result of elongation of A region. The other regions did not show significant changes in width during reopening.

When we compared equivalent stages of trap closure and reopening we did not find similar tissue to be involve thus, one was not anatomically a minor image of the other. Since reopening is also a highly coordinated event involving simultaneous events in both lobes this supports the possibility that there is an *open* signal like that of closure. Reopening may take up to 30-40 hrs and only the bulge occurs quickly [in about an hour]. Lea (1976) proposed that lysophosphatidic acid or similar compounds may be candidates for such an "open" signal.

One problem posed by a civil engineer I worked with was how stresses within the trap were relieved as trap morphology changes. A student (Bret Jankosky) working in my lab developed a model based on measurements of changes in cortical/medullarty cell positions relative to the epidermal cells along the entire length of the lobe at the open and sealed stage of closure (*i.e.* least and the greatest bend in the trap). His data supported the idea that cortical tissues might slide one against the other thus relieving stresses. We have more works to do on this but it is a intriguing possibility and is somewhat supported by the fact that the volume of intercellular space in these tissue is substantial.

Conclusions

1) Closure and reopening involves complex and coordinated changes in both inner and outer trap tissues from different trap regions at different times.
2) Morphological stages of closure and reopening appear to be minor images of each other, but not so with tissue dynamics associated with the changes in trap lobe shape.

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Open-closure trap system modeled in tissue-cultured *Dionaea muscipula* controled by certain chemical substances in culture media

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Abstract. Correlationships between certain macro-components of 1/2 strength of MS agar medium supplemented with no growth substance and growth, red-color pigment and closure and re-opening in traps were studied in cultured *Dionaea muscipula in vitro* generated from multiple shoots. The plants cultured in 1/2 strength of MS agar medium modified with 10.31mM NH_4NO_3 and 9.40 mM KNO_3 but no other macro-component and supplemented with 0.75 or 0% sucrose continuously proliferated by multiple shoots and generated large, green-colored plants, while those with dilution of those nitrogen components to 1/2 down to 1/8 and increase of sucrose up to 1.5% under continuous light exposure did not much proliferate but formed better carnivorous condition in relatively inverse proportion to depths of red-color anthocyanin pigmentation spread from glands to the upper surface of the trap, to plant sizes and dry weight and moreover trap healthy movement. If one or two plants, with four to five leaves by removal of old leaves, originated from big masses of multiple-shoot plantlets were placed in low population density on the solid medium, they got produce bigger leaves with trap. Deposit of he anthocyanin pigments of *Dionaea muscipula* consisted of delphinidin 3-0-glucoside and cyanidin 3-0-glucoside (chrysanthemin) was an index of trap movement.

Introduction

Dionaea muscipula Ellis, the Droseraceae is the king of carnivorous plants and scientifically and ornamentally very meritorious resource (Kondo and Kondo, 1983). The species is endemic to North and South Carolinas of the U.S.A. (Kondo and Kondo, 1983). The species has commonly dark red, red, yellowish red, yellowish green to green color in plant body in wild, cultivated and even *in vitro* culture conditions. These differences in color pigmentations may be correlated with differences in amounts of nutrient uptake from prey or soil, soil pH, light intensities or other environmental factors in habitats or laboratory and in genetic diversity within the species according to our experiences and may be furthermore correlated with trap movement (Kondo, unpublished).

Plants of *D. muscipula* which have stable red color are horticulturally more meritorious than those which have green color. Some strains of the species artificially

*Dedicated to Prof. Emeritus C. Ritchie Bell, The University of North Carolina, Chapel Hill, and Tokuyoshi (Masahiro) Kondo, KK's late father.

selected contain stabilized deep red pigment in the whole plant body *in vivo* cultivated condition to be the commercialized cultivars of 'Red Dragon', 'Red Giant', 'Red Purple', and 'Royal Red'. However, these cultivars can be artificially removed red pigment and kept green color by adjusting some medium components and light intensity *in vitro* culture (Kondo unpublished).

Dionaea muscipula, which is eutrophic plants containing chlorophyll in their leaves for photosynthesis, commonly occupy sunny, open, relatively closed ecosystem where the soil is poor in nutrient substances, wet and acid and intake and absorb nutrients directly from insect or small animal resources by insect-eating mechanisms differentiated in leaves (Lloyd, 1942). However, under shade conditions or at low CO_2 availability, the resultant negative photosynthetic benefits in those species as well as the other carnivorous plants are counterbalanced by organic carbon uptake from prey (Adamec, 1997). Darwin (1878, 1899) and some other workers (Kellermann and Raumer, 1878; Thum, 1988, 1989; Gibson, 1991) experimented and stated that the plants of some *Drosera* species fed artificially meat, aphids, or fruit flies increased numbers of flowers, total weight of seeds and winter buds, or dry weights of summer and winter plant, more leaves, and a larger trapping area. Thus, animal food supply is an important limiting factor for the *Drosera* species in the field (Thum, 1988, 1989; Gibson, 1991). However, the plants of *Dionaea muscipula* fed and examined mineral nutrients grew poorly, while the non-fertilizing controls grew prominently (Robert and Oosting, 1958; Juniper *et al.*, 1989). Studies on correlationships between nutrients, anthocyanin color pigmentation and moreover trap movement in carnivorous plants are lacking. The culture of another carnivorous *Utricularia praelonga* St. Hil. (Idei and Kondo, 1998) showed different organogeneses, micropropagation, growth form, and so on by adjusting KNO_3 concentrations between 24.73 and 3 mM as well as BAP (N_6-benzylaminopurine) concentrations in B5 (Gamborg *et al.* medium) liquid medium (Gamborg *et al.*, 1968).

Since these differentiations can be correlated with differences in nutrient substances in habitats, *Dionaea muscipula* as well as many other carnivorous plants can be cultured in a closed, well-controlled microenvironment *in vitro* to study the relationships between growth and micropropagation habits, trap closure and re-opening and pigmentation and specific chemical components of the medium. These data would also contribute to satisfactory ecophysiological treatment of the carnivorous plants.

Materials and Methods

Plant Materials - Individual plantlets of *Dionaea muscipula* both erect and rosette forms used as explants *in vitro* were planted on basal, 1/2, 1/4 and 1/8 strengths of MS (MS, 1/2 MS, 1/4 and 1/8; Murashige and Skoog) medium (Murashige and Skoog, 1962) supplemented with 0, 0.75, and 1.5% sucrose on each 80 ml medium supplemented with no growth regulator at pH 5.6 in each culture vial 58X80X129.5 mm in size and were placed at 25℃under 3500 lux continuous illumination. After five months they propagated average six plants per explant by adventitious buds and multiple shoots.

Each plant with 4-5 leaves and traps ca 1 cm long was used for the present experiment.

Effects of the macro-element of 1/2 MS and its diluents and sucrose concentrations on growth, trap closure and re-opening and color pigmentation - Plants of *D. muscipula in vitro* obtained were utilized to study effects of the macro-element at 1, 1/2, 1/4, 1/8 and 1/16 strengths or 0 in 1/2 MS medium and sucrose at concentrations of 0, 0.75, and 1.5% on growth, trap closure and re-opening and color pigmentations.

Effects of five macro-components in 1/2 MS on growth, trap closure and re-opening and color pigmentation - Plants of *D. muscipula in vitro* were also utilized to study effects of the five macro-components in 1/2 MS, such as NH_4NO_3, KNO_3, $CaCl_2$, $MgSO_4 \cdot 7H_2O$ and KH_2PO_4, by remaining or removing, on growth, trap closure and re-opening and color pigmentation.

Visible color identification in plant bodies - Identification of visible coloration on plant bodies caused followed the Japan Color Standard for Horticultural Plants (Japan Color Research Institute).

Detection of anthocyanin - Every 0.2 g fresh weight of plant bodies easpecially leaves per sample was utilized to extract anthocyanin pigments with 1 ml MeOH-HCl mixture (methanol:hydrochloric acid=1000:1) for 3 h to overnight and filtrated by Toyopak ODS M (Tosoh) and Maisyoridisc H-13-5 0.45 μ m (Tosoh) pre-cartridge. Following Iwashina (1996), the composition of plant extracts was determined by HPLC (high performance liquid chromatography; JASCO HPLC System) with the Model 880-51, two-line degasser and Syringe loading sample injector 25 Model 7125 (Rheodyne Inc.). Multi channel UV-visible detector Multi-330 connected with a computer was used to record the chromatogram data and UV-visible spectra. The dimension of the column was 120 X I.D.4 mm (Tosoh TSK-Gel, ODS-80TM). Samples (10 μ l each) were injected and eluted with H_3PO_4-AcOH-CH3CN-H2O (3:8:6:83) at a flow rate of 1.0 ml/min at a pressure of 99-106 Kg/cm^2. Compounds were detected at 360-660 nm for the presence of anthocyanin.

Identification of the anthocyanins contained in the two species was made by HPLC comparisons with authentic samples. For double identification of the anthocyanin bands separated from the crude extracts with BAW (n-BuOH-HOAc-H2O, 4:1:5, upper layer) by mass-PC (paper chromatography) were redissolved in 0.01% MeOH-HCl. After they were concentrated, they were eluted and separated using Sephadex LH-20 column with 70% MeOH and a drop of HCl, and purified individual anthocyanins. UV (ultra violet) spectra were measured by an UV spectrophotometer. Those anthocyanins were directly hydrolyzed in 12% HCl for 3 min on heating, cooled down, added and shaked with isoamyl alcohol to separate anthocyanidin in alcohol layer and glucose in water layer. Those anthocyanidin and sugars were identified by PC comparisons with their authentic samples. PC and UV spectral data of isolated anthocyanins were listed as follows: Pelargonidin 3-0-glucoside. PC: Rf 0.49 (BAW), 0.12 (1% HCl), 0.60 (FAH=formic acid:HCl:H_2O, 5:1:4), 0.36 (AAH=HOAc:HCl:H_2O, 15:3:82); color (visible)-orange. UV: 0.01% MeOH-HCl max 511 nm; +$AlCl_3$ $\triangle\lambda$ 0 nm; E440/Emax=33.6%. Pelargonidin 3-0-galactoside. PC: Rf 0.36 (BAW), 0.12

(1% HCl), 0.62 (FAH), 0.37 (AAH); color (visible)-orange. UV λ max 0.01% MeOH-HCl 5ll nm; +$AlCl_3$ $\triangle\lambda$ 0 nm; E440/Emax=33.6%. Cyanidin 3-0-glucoside. PC: Rf 0.27 (BAW), 0.06(1% HCl), 0.50 (FAH), 0.26 (AAH); color (visible)-purplish red. UV: λ max0.01% MeOH-HCl 529 nm; +$AlCl_3$ $\triangle\lambda$ 44 nm; E440/Emax=23.8%. Cyanidin 3-0-galactoside: PC: Rf 0.24 (BAW), 0.06 (1% HCl), 0.50 (FAH), 0.26 (AAH); color (visible)-purplish red. UV: λ max0.01% MeOH-HCl 530 nm; +$AlCl_3$ $\triangle\lambda$ 43 nm; E440/Emax=24.8%. Cyanidin 3,5-di-O-glucoside. PC:Rf 0.17 (BAW), 0.16 (1% HCl), 0.67 (FAH), 0.42 (AAH); color (visible)-purplish red. UV: λ max0.01% MeOH-HCl 522 nm. Delphinidin 3-0-glucoside. PC: Rf 0.19 (BAW), 0.03 (1% HCl), 0.39 (FAH), 0.17 (AAH); color (visible)-reddish purple. UV: λ max0.01% MeOH-HCl 540 nm; +$AlCl_3$ $\triangle\lambda$ 47 nm; E440/Emax=22.3%

Results and Discussion

Traps got much larger if one or two plants with four to five leaves each were isolated from a mother mass of plantles generated by multiple shoots or precocious branching and were conditioned in very low population density. Additionally, removal of old leaves from plant body *in vitro* could be another factor to make the traps larger.

Modified 1/2 to 1/8 MS media with less or no macro-element and with more sucrose induced red-color pigmentation in the upper surface of trap lobe and furthermore in the whole leaves in *Dionaea muscipula* after four months culture (Ichiishi *et al.*, 1999). They had trap closed if their trigger hair was touched and stimulated. However, they made plant growth slow down. In contrast, 1/2 MS media with more to complete macro-elements in absolute strength promoted deeper green colored in the whole plant bodies and larger growth and more proliferation of plantlets by multiple shoots or precocious branching.

Moreover, the modified 1/2 MS media with no NH_4NO_3 performed a little red colored glands, glandular tissues and trigger hairs on the upper surface of the trap but green colored in the lower surface of the leaves of the species after four months cultivation and the modified 1/2 MS medium with no NH_4NO_3 and no KNO_3 performed red coloration in the upper surface of the trap lobe and relatively red color in the whole leaves and reduction of dry weight. These red-colored traps could be made themselves closed and reopened if their trigger hairs were artificially touched twice or more. Too large traps as well as too small traps in the plant *in vitro* did not show trap movement although the trigger hair was stimulated; traps average 1 cm long were just right size for active trap movement. A natural habitat of *D. muscipula* in North Carolina, U.S.A. had low contents of NH_4^+ (2 mg/Kg dry weight), PO_4 (less than 2 mg/Kg), K (2 mg/Kg) and Mg (1 mg/Kg) and no content of NO_3^-, Ca and Mn (Robert and Oosting, 1958). The plants in the natural habitat of the species showed commonly red color in the upper surface of the trap lobe and yellowish green in the other lower surface. On the other hand, the lack of NH_4NO_3 and $MgSO_4 \cdot 7H_2O$ among the macro-components of the 1/2 MS medium exhibited healthy-looking plant bodies without any dead leaf but no plant

growth perhaps due to balanced combination of N, P, K, and Ca. In contrast, the lack of $CaCl_2$, $MgSO_4 \cdot 7H_2O$ and KH_2PO_4 among the macro-components of the 1/2 MS medium treated the leaf and shoot tips dead perhaps due to unbalanced combination of less Ca against more N and K.

The anthocyanin pigments of *Dionaea muscipula* consisted of delphinidin 3-0-glucoside and cyanidin 3-0-glucoside (chrysanthemin).

Thus, dilution of NH_4NO_3 and KNO_3 down to 1/2 to 1/16 strengths in volume and increase of sucrose up to 1.5% in the 1/2 strength of MS medium and continuous light illumination mainly promoted and could be in relatively inverse proportion to depths of red-color anthocyanin pigmentation spread from glands to entire leaves of the species and best trap closure and re-opening.

Insects contain total nutrients of N (99-121 g/kg dry weight), P (6-14.7 g/kg), K (1.5-31.8 g/kg), Ca (22.5 g/kg) and Mg (0.94 g/kg) (Reichle *et al.*, 1969; Watson *et al.*, 1982; Dixon *et al.*, 1980) that are somewhat smilar to the medium requirements studied here. In nature, preys would be more atractive and captured to red-colored plants than to green-colored ones in the species. Generally speaking, if plants get heavy stress of temperature, physical damage of organ, lack of nitrogen and phosphoric acid, light, or other environmental factors, they often show anthocyanin color pigmentation (Harborne and Grayer, 1988). *Dionaea muscipula* as well as other carnivorous plants could have adaptation strategies to barren, wet and low pH soil condition by interaction between leaf carnivory and low root nutrient especially both NH_4+ and NO_3^- depended greatly on pH (Adamec, 1997). The present examination in certain microenvironments in tissue culture supports that the species would turn red color when they got deficient in nitrogen compounds to make the prey attractive and would catch more preys as leaf carnivory if they had too low root nutrient to survive, grow and propagate. The anthocyanin pigmentation in the two species may make it possible to be biosensor against nitrogen consumption uptake and furthermore degree of active movement of trap.

This methodology in culture medium has been partially patent pending by the patentee Bioparco Sanmei.

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Chemical substance concerning the leaf-movement of *Dionaea muscipula*

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Abstract. Some internal bioactive substance would be concerned in the closure of traps in *Dionaea muscipula*. We showed that bioactive substance inducing the closure of traps in contained in the extract of *D. muscipula*, and purification of this substance is reported.

Introduction

Venus's Flytrap (*Dionaea muscipula*) is known as representative carnivorous plant (Fig. 1). This plant traps the insects by its large leaves called trap, and digested them between the traps by digestive enzymes. It is known that Charles Darwin studied this plant extensively, and he called this plant one of the most wonderful in the world (Darwin, 1875). The rapid movement of the traps requires twice stimuli on their sensory hairs which exist on the internal surface of the traps. There observed some "memory" in this biological event. Many studies with electrophysiological method was carried out and gave important information on this biological event. Many studies with electrophsiological method was carried out and gave important information on this biological event (Burdon-Sunderson, 1873, 1899; Jacobson, 1965). However, chemical studies concerning the internal bioactive substance which induce the closure of traps was carried out. We thought some bioactive substance would be involved in this "memory". If some bioactive substance was secreted stepwise in the plant body by two stimuli, the "memory" can be interpreted by the accumulation of the secreted bioactive substance. Thus, we tried to isolate the internal bioactive substance which induce the closure of traps in *D. muscipula*.

Material and Methods

General notes. 2D-NMR, ^{1}H-NMR (400 MHz) were recorded with a Jeol JNM-A400 spectrometer in D_2O, using *t*-BuOH as an internal standard [^{1}H-NMR (d 1.23)] at room temperatures. HPLC analyses were carried out with a Jasco PU-960 pump equipped with a UV-970 detector to monitor the UV absorption at 220 nm. All the solvents used for HPLC were obtained from Kanto Chemical Co. and were filtered through a Toyo Roshi membrane filter (cellulose acetate of 0.45 mm pore size, *f* 47 mm) before use.

Plant material.. Leaves of *D. muscipula* were collected in August 2000 which was purchased from Yoneyama Plantation, Yokohama, Japan. *D. muscipula* used for the bioassay grown in a biotron (BIOTRON NK-300, NK-system Co, Ltd) using modified MS (Murashige-Skoog) medium[5] at Keio University under continuous light conditions with 60% humidity at 27 °C.

Bioassay Young leaves were detached from the stem of *D. muscipula* plant with a sharp razor blade and used for the bioassay. One leaf each was placed in H_2O in glass tubes, in a biotron at 25-30 °C. The sample solution was carefully poured into the glass tube to attain appropriate concentration. The bioactive fraction made the leaf of *D. muscipula* close within 50 hours.

Purification of bioactive substance from the extract of *D. muscipula*. Fresh whole leaves of *D. muscipula* (777 g) were collected and chopped by scissors and then extracted with 10% aqueous methanol (4 l) at 4 °C for one week. The extract was concentrated *in vacuo*, and centrifuged

(10000 × g, 20 min, 4 °C) to remove insoluble. Then the solution was partitioned with *n*-hexane, ethyl acetate, and *n*-butanol. The bioactive aqueous phase was then subjected to ultrafiltration with Centricon YM-10 (2000 ¥ g, 90 min ¥ 2, 4 °C), and then with Centricon YM-3 (2000 × g, 90 min × 2, 4 °C). And bioactive fraction was separated by gel filtration column chromatography using TOYOPEARL HW-40 (*f* 50 × 500, 20% 1.0 ml/min) to give two bioactive fractions: high-molecular weight (9.5 mg) and low molecular weight (11.7 mg). The high molecular weight bioactive fraction was also subjected to anion-exchange column chromatography (Hi-prep16/10 Q XL, Tris-HCl buffer, pH 8.0). Elution was carried out by using 1.0 M NaClaq, and the eluted fraction was desalted with Hi-prep 16/10 Desalting column.

Results and Discussion

Purification of bioactive substance was carried out according to the result of bioassay using the leaves of *D. muscipula* which was cultured by tissue culture using modified MS medium. The use of genetically uniform stain gave high reproducibility to the bioassay.

The leaves of *D. muscipula* was extracted with 10% aqueous methanol (4 l) for a week at 4 °C to circumvent the decomposition of the bioactive substance. After centrifugation and partition with organic solvents, bioactivity was monitored in the aqueous phase. No organic phase showed bioactivity. Thus, bioactive substance will be a water-soluble compound with high polarity. The ultrafiltration using Centricon YM-10 and YM-3 gave two bioactive fractions, one is a fraction with molecular weight of 10000-3000, and the other is a fraction with molecular weight below 3000. Both of fractions were bioactive at 5 g/l. The aqueous phase after partition with organic solvents was also purified by repeated gel filtration using TOYOPEARL HW-40 to give two bioactive fractions with high and low molecular weight (Fig. 2). These results showed that two bioactive substance inducing leaf closure is contained in *D. muscipula.*

^{1}H NMR spectrum of high-molecular weight fraction gave very broad signals among 3-4 ppm (Fig. 2). Thus, the main component of this fraction was estimated to be some polysaccharides. And after anion-exchange chromatography of a fraction with molecular weight of 10000-3000, the bioactivity (10 g/l) was observed in the fraction which was eluted with 1.0 M NaClaq. ^{1}H NMR spectrum of the eluted fraction gave very broad signals among 3-4 ppm, which is closely related to the one of high-molecular weight fraction after gel filtration. These results strongly suggested that bioactive substance in high-molecular weight fraction is acidic polysaccharide which contain some uronic acids in the molecule. We examined several commercially available anion-exchange column, thus MonoQ HR 5/5 was found to be very effective for the purification of bioactive substance. The purification using this column is now in progress.

On the other hand, ^{1}H NMR spectrum of low-molecular weight fraction after gel filtration gave very complex spectrum (Fig. 2). Thus, we will examine further purification with HPLC on this fraction.

We found that *D. muscipula* has some internal bioactive substance which induce the closure of traps without stimuli. These bioactive substances would be clue for the interpretation of the "memory" observed in the movement of *D. muscipula* on molecular level.

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Figure 1. Venus's Flytrap (*Dionaea muscipula*)

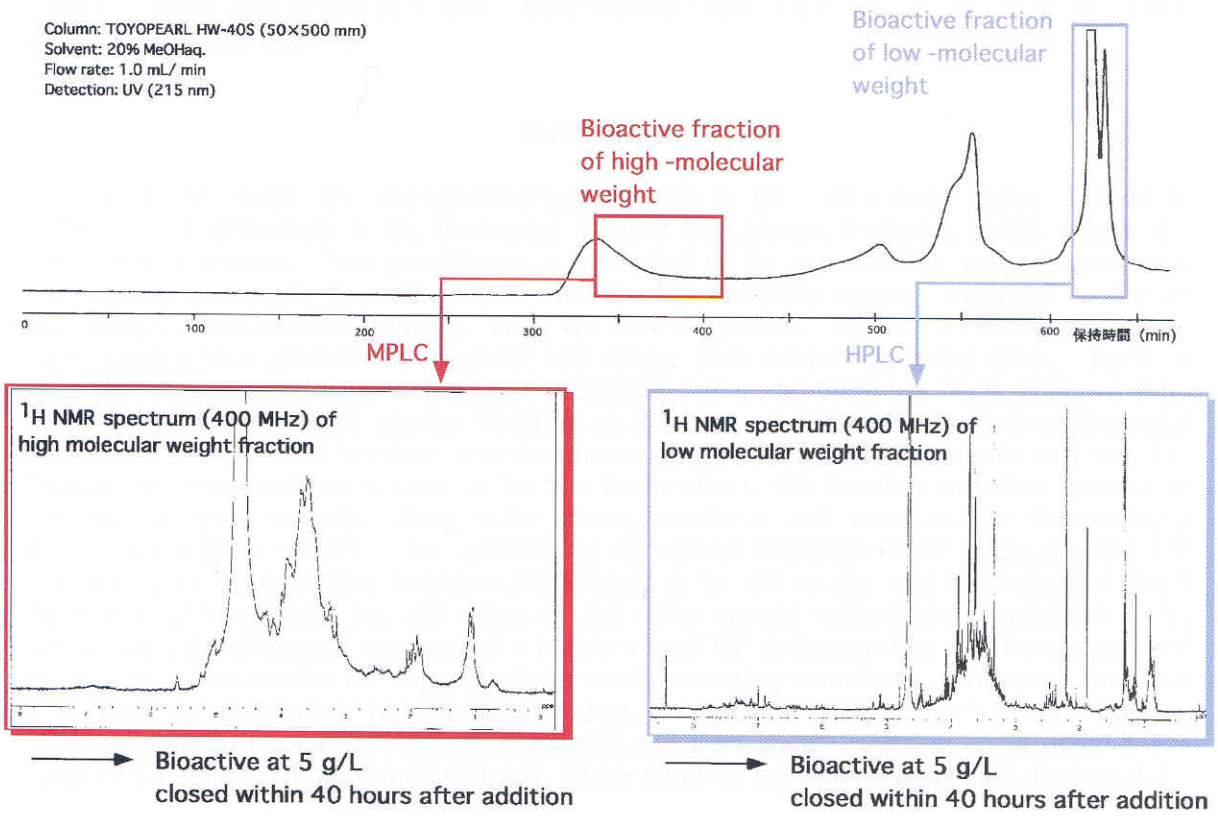

Figure 2. Purification of bioactive substances inducing the closure of the traps of *D. muscipula*.

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The carnivorous plant collection at California State University, Fullerton: An exercise in relevance

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Introduction

This presentation is to showcase the extensive carnivorous plant (CP) collection at California State University, Fullerton (CSUF) that I curate by describing in detail the collection we house, and by placing the collection into a broader context. This context includes three arenas:

1. The university, and all the public and private educational institutions that relate to the Cal State system;
2. The local and state-wide geographical community (especially cities on the West Coast); and
3. Horticultural interests.

The CP collection at CSUF has stand-alone merit, but its importance is also embedded in a field of actual and potential users. Determining these functions is the point of doing presentations like this one.

Background

In order to discuss the overall collection as well as the carnivorous plants, I want to establish a brief history of the Biological Science Greenhouse Complex (GH), where the collection is housed. This greenhouse is a product of the considerable support generated for science teaching by Sputnik, a time when the US decided it had to "catch up" to Soviet technological and science training. Thus we have a spacious, .66-acre/1.66 hectare facility that includes four greenhouses and one lath house, plus outdoor growing areas. There is also support for a full-time well-trained technician (me), and there has been plenty of time since my hire thirty years ago for building an extensive and varied collection of botanical materials. I've had the freedom over the course of my career to do essentially as I saw fit. Besides the huge collection (one of the best in the state), this freedom has also resulted in my having time to help, along with others, establish and maintain the International Carnivorous Plant Society. So, networking became an important factor in creating the CP collection; we more or less found a critical mass at the university, and the collection itself became highly singular (as did some of the other special collections I maintain). An unexpected development: we created a highly varied CP collection that has far outstripped the needs of the faculty for support of day-to-day teaching exercises. (Note: we are not a PhD-granting institution, but our faculty does do research with master's level students. Also of note: in the late 1970s, the university and the City of Fullerton constructed a 15-acre/37.5-hectare Arboretum on campus, where some of my mature plants are displayed.)

The Problem

The situation, in the days of diminished educational resources, has forced me to justify the whole GH collection, especially in light of the time it takes to support the significant variety of plants we have under glass and lath, and within the GH area. In response, I've worked in three areas:

1. I've upgraded the facility;
2. I've worked on integrating the entire collection with faculty and with the Arboretum; and
3. I've articulated a mission that allows me to judge critically the collection as a whole, so that I can make decisions about what to keep and what not to keep at the GH.

I can best describe this mission as triangular in nature (See Fig 1). Developing the horticultural and teaching elements of this mission are relatively easy tasks. That is, I can identify horticultural and teaching uses of most of the plants in the GH. Research uses are less obvious. And, the overall problem - - what plants best serve the sophisticated GH mission of this campus within the California State University system - - is also difficult to resolve.

The Plant Material

Looking at the plants demonstrates these difficulties. I keep several categories of CPs at the GH, for example. (Here, I will give a brief overview and slide show of the entire collection plus, I will describe pings, traps, suns, border plants and related oddballs. I will also show how the *Sarracenia* and the *Nepenthes* in particular demonstrate why I'm under pressure to justify the CP collection.

Specifically: I have 40 Sarracenias in pots that represent 10 species in their various forms. But I also have approximately 360 *Sarracenia* hybrids, so in effect, 90% of the collection could be considered redundant. However, in this very personal collection there are many plants that are mine, plants that I've developed and named and are fascinating to other CP hobbyists. I've used this collection, incidentally, for all kinds of interesting outreach, including in movies and on TV. And I don't think the hybrids are redundant: to me, they demonstrate horticultural possibilities and in teaching, taxonomic variations that may or may not be possible to see in nature. (Research potential hasn't caught on yet. This will take some work.)

However, the extensive nature of the *Sarracenia* and the *Nepenthes* collection, too, is exactly what causes confusion on campus about what the collection means. Do we need all these plants when the faculty doesn't use even a small percentage of them in the classrooms? Why do we have so many hybrids . . . how can I justify using my limited time and attention to care for them? There aren't fellow botanists on campus with whom I can share these concerns. Developing a full complement of horticultural, teaching and research possibilities for the *Sarracenia* and the *Nepenthes* is a problem I'm more or less tackling alone. And, my creating a public awareness of these issues (remember, CSUF is an institution supported by taxpayers) has in fact created some contentions about use of campus resources. Plus, I'm not sure at this time what the future of the GH holds for the CPs.

Strategies

What can I do to insure the survival of this most important special collection? I've developed a 5-part, 5-year plan that includes the time from now until my retirement at about age 66.

1. My first strategy is to preserve and even add to the whole GH collection, especially the CPs, until the matter is finally resolved with my leaving.
2. I'll propagate and disseminate the collection, but also look at each plant for its contribution to a integrated facility.
3. I'll continue to work on the facility, that is, upgrading the buildings and grounds (cataloguing, signage, displays, landscaping, etc.) and working with faculty and administration to secure the use of the plants.
4. I will work hand-in-hand with the Arboretum, and this is perhaps the key to preserving the CP collection intact. Besides moving large plants to the Arboretum, my aim may very well be to develop and sustain a conservancy, that is, to use the CP and my other special collections as a core of a conservatory display. This will require, of course, space, funds, personnel, and a general public awareness of the importance of the collections that doesn't now exist. This strategy is still very much in the earliest stages of its development . . . I can't say much about its eventual efficacy.
5. Finally, an alternative to keeping the CP collection at CSUF is to move it out in its entirety to another institution, such as (perhaps) the Huntington. And I myself will move, too.
6.

In closing

In other institutions, these sorts of questions and answers are not the prerogative of technicians. But at CSUF, I have developed a certain degree of authority, mainly because of the CPs, my reputation as a collector and as someone who can grow the plants. This has allowed me to cultivate leadership at the university and within the community; I'm able to take on a more public role than most of my peers at work. In fact, I've actually changed the informal definition of my job. Where once I may have been considered solely as "class support," now I've become much more a curator of plants, a much more sophisticated and independent position. Advocacy and administration is a new situation for me, and is taking up a substantial bit of my time.

Yet, I also want to stay intimately involved with the care of the plants. I like the feel and the touch, the experience of dealing with new material, seeing it grow and getting it to mature and flower. I love giving the plants away to people who I know will use and care for them. I get a thrill from talking with students, hobbiests, peers and the public in general about the fascinating complexities of the GH and the botanical world it represents. So, the question for me is, Can I accomplish the changes the GH, the CPs, require at CSUF to remain an integrated and well-appreciated resource on campus? I don't know. That's why I will be in Tokyo: to share these concerns with you and see if you have any responses to them.

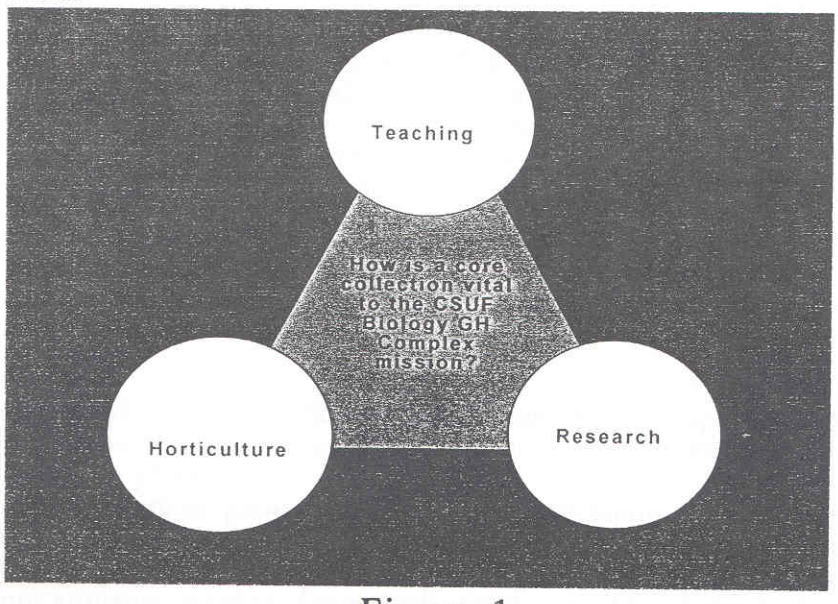

Figure 1

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Recent observations on the genus *Heliamphora* (Sarraceniaceae)

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Abstract. Over the last years we had the opportunity to visit some of the least known of the remote table mountains of the Venezuelan south, the so-called 'Tepuis'. During these visits we made several interesting findings including new *Heliamphora* species and could also gain a better understanding of the species already known.

Introduction

The genus *Heliamphora* is one of three genera of the family *Sarraceniaceae*. Currently *Heliamphora* consists of seven species generally accepted and at least two more await publication.

Most species of Heliamphora are growing on the flat tops of a group of remote and isolated table-mountains 2500 to 3000 m high with almost vertical walls, so-called Tepuis. These sandstone mountains are located in the Guayana Highlands of Venezuela near the Borders to Brazil and Guayana.

Many of them have just been explored in recent years, some not at all so far. Due to isolation, a lot of endemic plants and animals, among them the different species of the genus *Heliamphora*, could evolve on their plateaus.

Over the last years, together with some fellow biologists, I had the opportunity to visit several of the remote Venezuelan Tepuis and study the species of *Heliamphora* growing on top, on the foothills and on the faces of these strange and ancient mountains.

Located in the north of the Gran Sabana northwest of Ptari Tepui and east of Auyan Tepui are four table mountains of the Aparaman-group ('Los Testigos'): Aparaman Tepui, Murosipan Tepui, Tereke-Yuren Tepui and Kamarkaiwaran Tepui (Steyermark, 1986; George, 1988). The altitude of these tepuis lies between 2100 meters (Tereke-Yuren) and 2700 meters (Kamarkaiwaran).

We also explored parts of the 'Macizo de Chimanta', the Chimanta Massif in the southwest part of the Gran Sabana. This huge massif covers a total area of 1470 km^2 and is actually a cluster of tepuis including the central Chimanta Tepui itself. Their peaks range in altitude from 1700 m (at the central part of the massif) to 2698 m (on Eruoda Tepui). The ten tepuis that reach 2000 m above sea level cover an area of some 700 km^2 in total (Huber, 1992). The size and the diverse altitudes of the Chimanta Massif support numerous habitats including rivers, green valleys and forests as well as rocky plateaus and moist savannas. Starting in the 1950s, various expeditions explored the unique flora and fauna of this area. Many endemic plants and animals were discovered during these expeditions and certainly many more await discovery.

Other mountains we visited are *Kukenam Tepui, Roraima Tepui, Ilu Tepui, Aprada Tepui, Tramen Tepui, Juruani Tepui* and the extremely remote *Cerro Neblina*.

Results

Last year we published one new species of *Heliamphora* from the Aparaman-group of Tepuis and have just recently submitted another publication of a new species from Chimanta Tepui. This paper currently is in press.

***Heliamphora* on Aparaman-group of Tepuis** On all tepuis of the Aparaman-group we found a very interesting, and previously undescribed species of *Heliamphora* notable for the unique anatomy of its pitcher-appendage. The appendage is fused with the back of the pitcher forming a hollow structure running downwards on the outside of the back of the pitcher,

apparently working as a chamber to store nectar produced by the glands of the appendage. Such a structure has never before been observed in *Heliamphora* or any other member of Sarraceniaceae.

We named this new species Heliamphora folliculata (Wistuba *et al.*, 2001). The name '*folliculata*' was chosen to highlight the 'bubble' (=follicle) formed by the nectar spoon, the most distinct characteristic of this species.

We did not observe any other species of *Heliamphora* on any of the Tepuis belonging to the Aparaman-group, however we observed *Heliamphora folliculata* on all four mountains: Aparaman Tepui, Murosipan Tepui, Tereke Tepui and Kamakeiwaran Tepui.

The two other tepuis in the area (Auyan Tepui and Ptari Tepui) are well explored. They are known to house two distinct species (*Heliamphora minor* on Auyan Tepui, and *Heliamphora heterodoxa* on Ptari Tepui) (Maguire, 1978; Steyermark, 1984). Accordingly, we believe that *Heliamphora folliculata* does not occur on these tepuis, and instead is endemic to the table-tops of the Aparaman-group.

***Heliamphora* on Chimanta Tepui** During our exploration of the Chimanta Massif we found a previously undescribed species of *Heliamphora* most notable for its pitcher shape and the spoon shaped lids.

The pitchers are infundibulate in the lower half, slightly ventricose in the middle and cylindrical to slightly infundibulate in the upper third. The lids are spoon-shaped, upright and end with a sharp tip. The two lobes of the lid are compressed from the sides near the tip, often touching each other at the front, forming a quasi-helmet. The lobes are expanded in the lower part of the lid and narrowed sharply near the base. Interestingly the inner side of the lid carry prominent irregularly shaped patches of glands which measure up to 5 mm across.

In comparison to the species found on the plateaus of the various tepuis, where the surface usually is much more rocky and sandy and plants often can grow only on 'islands' of debris, highly limited in space, the moist savannah-like habitat *H. chimantensis* prefers allows the formation of huge clumps. We have visited many tepuis on this and other expeditions, and had never before seen clumped *Heliamphora* colonies of comparable in size to the ones typical of *H. chimantensis*. Vegetative reproduction seems to play an important role as the seedling activity we observed was very low.

The discovery of this species on Chimanta which, by now, seems to be restricted to Chimanta Tepui and its characteristics came as quite a surprise, as they clearly indicate that it is much more closely related to the southern *H. tatei*, than to any of the northern species known to be growing in the Gran Sabana. The flowers of all other species known from the Gran Sabana have 10-15 anthers, while the new species as well as *Heliamphora tatei* var. *tatei* and *H. tatei var. neblinae* from the Amazon have about 20; however, while the anthers of *H. tatei* and *H. tatei var. neblinae* are 7-9 mm long, those of the new species from Chimanta just reach 5 mm in length (Maguire, 1978; Steyermark, 1984).

A paper is submitted and has been accepted for publication In Carnivorous Plant Newsletter (Wistuba *et al.*, 2002).

Two other taxa of *Heliamphora* have been recorded from Chimanta Tepui: *Heliamphora minor* and *Heliamphora heterodoxa var. exappendiculata*.

The *H. minor* plants on Chimanta are notable for the long and prominent bristles inside the pitchers. This variant form of *H. minor* is actually fairly widespread and has been found on many tepuis of the Chimanta Massif as well as on Aprada Tepui, but not Auyan Tepui. Meanwhile, the typical form of *H. minor* is only known from Auyan Tepui. The differences between these two forms of *H. minor* may indeed merit further taxonomic study. The new species sometimes grows together with *H. minor*. As a consequence, hybrids frequently could be found. However, *H. chimantensis* seems to prefer valleys growing at around 2000 m while *H. minor* also has been found in higher altitudes and usually prefers more open habitats.

While we found *Heliamphora heterodoxa var. exappendiculata* on Aprada Tepui, we could not locate it on Chimanta.

Discussion

During our studies we found the pitcher-lids or so called nectar-spoons of *Heliamphora* to be highly elaborate structures for the attraction of prey. Apparently various strategies have been developed by the different species of *Heliamphora* all using the lids in altered ways. The

diversity of the pitcher nectar spoon in the various species of *Heliamphora* - *H. minor* which has a highly differentiated helmet-like structure, *H. tatei* which has a rather primitive flag-like structure, *H. heterodoxa* var. *exappendiculata* with its rudimentary lid, and *H. folliculata* as described - illustrate the various ways the nectar spoon is shaped in order to function as the predominant structure for the attraction of prey (Jaffe *et al.*, 1995). Being morphologically fairly constant organs, the lids offer superb, yet previously under-utilized, characters of taxonomic relevance (Nerz and Wistuba, 2000; Wistuba *et al.*, 2001; Wistuba *et al.* 2002).

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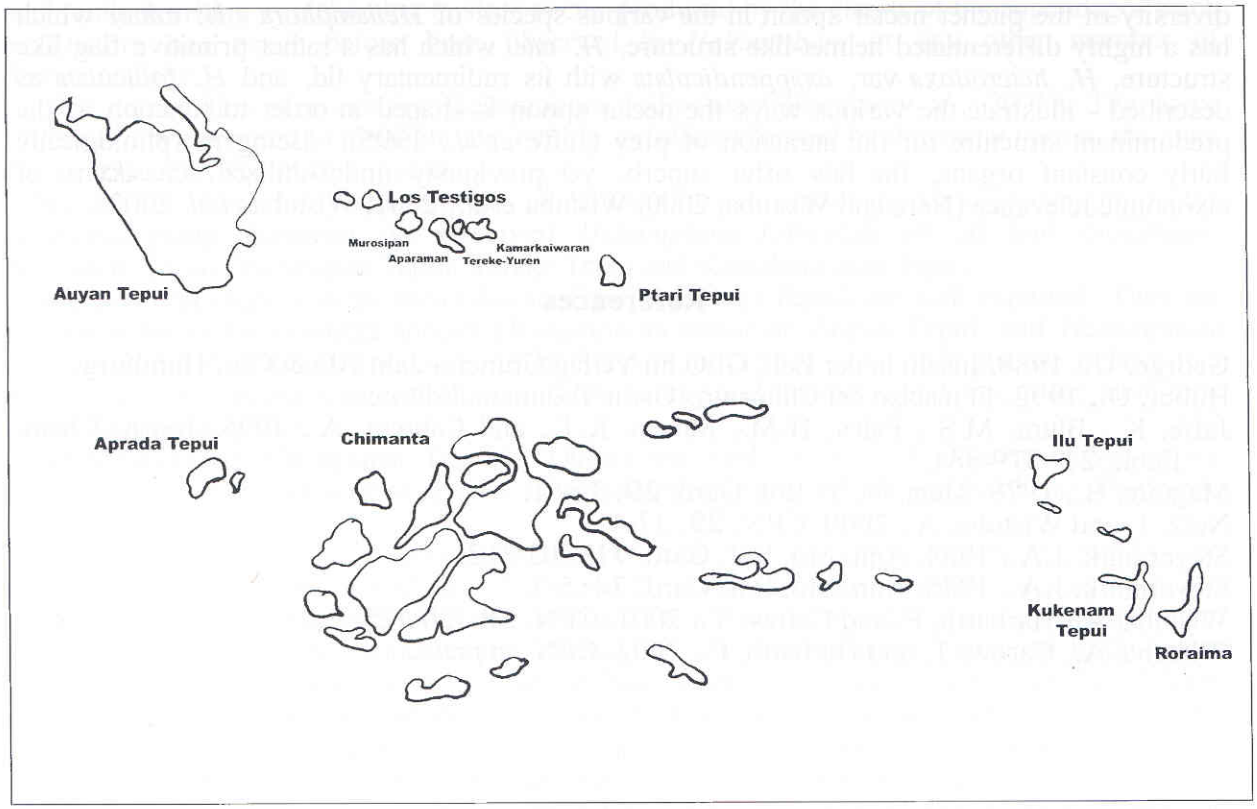

Fig.1 Map indicating the peaks in the complex of tepuis. The indicated peaks have elevations of more than 1500 meters higher than the surrounding plains.

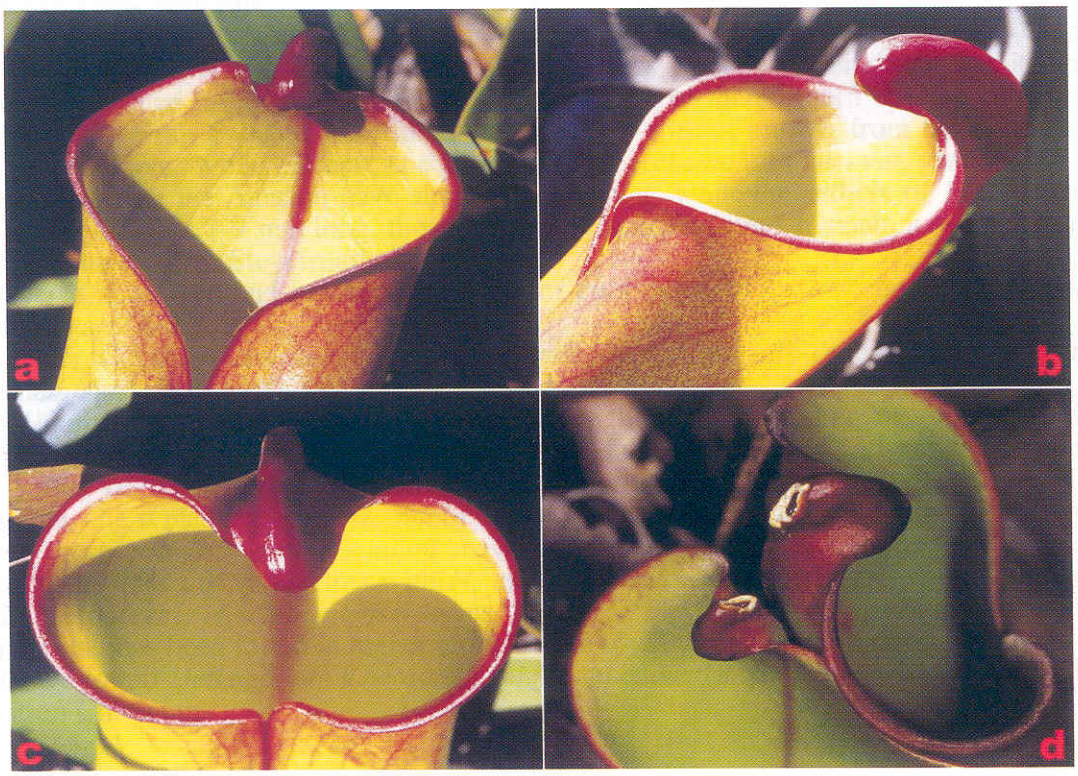

Fig.2 Details of *H. folliculata* pitchers.
a) Necatar running down from the lid (dark red).
b) Lid showing the hollow hunchback-like structure.
c) Pitcher being compressed between front and back leading to kidney shaped mouth.
d) Nectar reservoirs opened by insects.

Fig.3 *H. folliculata* pitchers on Aparaman Tepui.

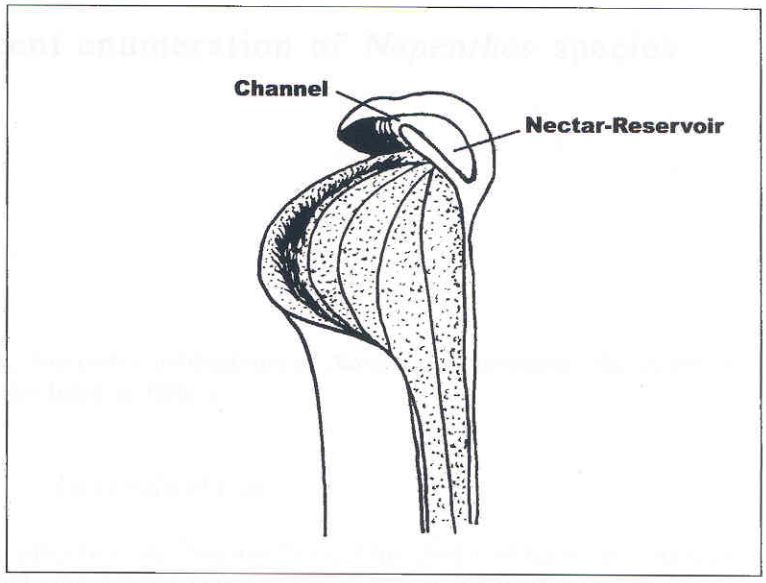

Fig.4 Drawing of the top of a *H. folliculata* pitcher in cross section showing the nectar channel under the lid appendage and the nectar reservoir

Fig.5 *Heliamphora spec.* nov. (publication submitted) from Chimanta Tepui -
a) Spoon-shaped lid with big nectar glands.
b)Group of plants.

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Revision trial in recent enumeration of *Nepenthes* species

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Abstract. On the basis of the recent, successive publications of *Nepenthes* monographs, the genus is estimated to consist of 91 distinct species listed in Table 1.

Introduction

Taxonomic treatment of species in *Nepenthes*, the Nepenthaceae rather not use stable sex organs but use vegetative organs such as leaf and pitcher with respect to morphology and structure supposedly unstable and variable caused by environmental changes in habitat. Flower characters of *Nepenthes* species are similar to each other. Standardization of taxonomic treatment is depended on which taxonomic characters are pointed out by authors or author's subjectivity. Thus, some distinct species are sometime treated as synonyms while some are in opposite opinions and some synonymous species and sometime changed ranking to independent species according to the presnt enumeration. Production of perfect list of taxonomically distinct species does not mean the summary result or thought of the *Nepenthes* taxonomists. Concerning these circumstances, the *Nepenthes* species described already by certain monographers after Macfarlane (1908) are here ventured to be revised and enumerated to see the whole view of the taxonomy of the genus since they have been taxonomically well revised and monographed and several new species of the genus have been described well these one decade.

Materials and Methods

In convenience, newly added and described species and eliminated species put into varietal or lower or synonym rankings in *Nepenthes* after Macfarlane's monograph (1908) were reviewed in literature information to meet the present trend of the species. The 57 distinct species Macfarlane (1908) described were put at the first step of study, while the synonymously treated species were put out of study excepting *N. fallax*, *N. hispida* and *N. sumatrana* later placed in the distinct species by some different workers were placed in the first step of study. Those species accepted as the distinct species by various workers after Macfarlane (1908) are designated by the symbol (O) and those species justified synonymy under certain distinct species are designated by the symbol (S) in the list table.

The literatures and summaries in contents used in this study are listed as follows:

1. Macfarlane, J. M. (1908). Nepenthaceae, *In*: A.Engler, Das Pflanzenreich, Ⅳ. 111: 1-92.
   Addition to the already-known 51 species in *Nepenthes*, seven new species were reported; total 58 species and two natural hybrids were

described. He stated that *N. gracillima* is very close relative of *N. albomarginata*. If they are conspecific, the number of distinct species could be 57. *Nepenthes fallax* is treated as a synonym of *N. maxima*, *N. hispida* is treated as a synonym of *N. hirsuta*, and *N. sumatrana* is treated as a synonym of *N. treubian*.

2. Danser, B. H., 1928. The Nepenthaceae of the Netherlands Indies. Bull. Jard. Bot. Buitenzorg. Ⅲ.9: 249-438.
He revised *Nepenthes* species distributed in the former Dutch Indies (present Indonesia), Malaysia and the Philippines and 58 species of the genus together with synomyms treated by Macfarlane (1908) into 39 species. He accepted and added later four new species described by Macfarlane, two new species described by Ridley, three new species described by Lecomte, and 17 new species he described, total 65 species including two natural hybrids that were divided into six groups. He treated *N. edwardsiana* as a synonym of *N. villosa* and *N. ramispina* as a synonym of *N. gracillima*.
(Note: Plants in New Guinea identified as *N. vieillardii* by him were later described as *N. lamii* by Jebb and Cheek)

3. Kurata, S., 1976. *Nepenthes* of Mount Kinabalu. Sabah National Parks Trust. Kota Kinabalu, Sabah.
He revised the species of *Nepenthes* of Mt. Kinabalu, Borneo and accepted 16 species (one undescribed species was later described as *N. macrovulgaris*). He made *N. edwardsiana* revived. Additionally, he reported the list of 71 species and one hybrid of *Nepenthes* of the world.

4. Tamin, R. and Hotta, M., 1986. *Nepenthes* di Sumatra: The genus *Nepenthes* of the Sumatra Island. *In*: M. Hotta, Ed., Diversity and dynamics of plant life in Sumatra. Kyoto Univ.
They revised the species of *Nepenthes* in Sumatra and described 17 species and one natural hybrid. They treated *Nepenthes carunculata*, *N. pectinata* and *N. spathulata* as synonyms of *N. singalana* and *N. inermis* and *N. dubia* as synonyms of *N. bongso*. He made *N. sumatrana* revived. They added new species of *N. adnata*, *N. rosulata* and *N. spinosa* (all *nomina nuda*).

5. Phillipps, A. and Lamb, A., 1996. Pitcher Plants of Borneo. Natural Hist. Publ. Kota Kinabalu, Sabah.
They revised the species of the genus in Borneo and accepted 32 distinct species, seven natural hybrids and one undescribed species.

6. Jebb, M. and Cheek, M., 1997. A skeletal revision of *Nepenthes* (Nepenthaceae). Blumea, 42: 1-106.
They listed total 85 species including three hybrids and pended *N. deaniana*, *N. neglacta* and *N. smilesii* as little known taxa. They excluded *N. cincta*, *N. cristata* and *N. lindleyana*. They treated *N. longifolia* as a synonym of *N. sumatrana*, *N. talangensis* as a synonym of *N. bongso* and *N. tenuis* as a synonym of *N. dubia*. They made *N. eustachya*, *N. pectinata* and *N. ramispina* revived.
They treated *N. faizaliana and N. sandakanensis* as synonyms of *N. stenophylla*, *N. xiphioides* as a synonym of *N. pectinata* and *N. carunculata* as a synonym of *N. bongso*. Furthermore, they added and described six new species such as *N. argentii*, *N. aristolochioides*, *N. danseri*, *N. diatas*, *N. lamii* and *N. murudensis*.

7. Schlauer, J., 2000. World Carnivorous Plant List. 'CP database (15 Nov. 2000 ed.).
He accepted 88 species in the genus. He made *N. fallax* revived and

treated *N. pectinata* as a synonym of *N. gymnamphora* or *N. singalana* and furthermore, *N. stenophylladescribed by Danswer as a synonym of N. fallax.*

8. Clarke, C., 1997. *Nepenthes* of Borneo. Natural Hist. Publ. Kota Kinabalu, Sabah.
   He revised the species of the genus in Borneo. He made *N. faizaliana* revived and accepted total 31 distinct species. He treated *N. borneensis as a synonym of N. boschiana.*
9. Clarke, C., 2001. *Nepenthes* of Sumatra and Peninsular Malaysia. Natural Hist. Publ. Kota Kinabalu, Sabah.
   He revised the species of the genus in Sumatra and Malay Peninsula and reported total 34 distinct species in which *N. angasanensis*was not well studied. He made *N. longifolia, N. talangensis and N. tenuis* revived and put *N. pectinata* as a synonym of *N. gymnanphora.* He described *N. jacquelineae* as a new species and recorded *N. sp* A and additionally, *N. sp* B as undescribed taxa. He treated *N. beccariana* as little known taxon.
10. Cheek, M. and Jebb, M., 2001. Nepenthaceae. *In*: Flora Malesiana, I. 15. National Herb. Nederland.
    They revised the species of *Nepenthes* in Malesiana including Malaysia, Indonesia, Brunei and Philippines and accepted 82 species including three natural hybrids. They made *N. pectinata* revived and treated *N. borneensis* as a synonym of *N. boschiana.* They treated *Nepenthes deaniana* as little known taxon and eliminated *N. cincta, N. cristata, N. lindleyana and N. neglecta.* They treated *N. angasanensis* as a synonym of *N. mikei* and *N. wilkiei* as a synonym of *N. philippinensis.* They added *N. benstonei, N. faizaliana, N. lavicola and N. mira* as another distinct species.
11. Kurata, S., 2001. Two new species of *Nepenthes* from Sumatra (Indonesia) and Mindanao (Philippines). Journ. Insectiv. Plt. Soc. 52: 30-40. (*in Japanese*)
    He described two new species *N. mindanaoensis* from the Philippines and *N. pyriformis* from Sumatra. However, he rather removed the latter species from the present list of distinct species since Clarke (2001) commented and pointed out that the latter species might be a natural hybrid.

Conclusion

It could be reasonable that *Nepenthes* consists of 91 distinct species listed in Italic in the summary table. However, this tabulation of the distinct species of *Nepenthes* is only an integrated, speculated conclusion based on the standard references. Many more studies based on total field observation, herbarium works and experimental researches are expected to clarify and justify the final number of species of *Nepenthes.*

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	Macfarlane (1908)	Danser (1928)	Kurata (1976)	Tamin & Hotta (1986)	Phillipps &Lamb (1996)	Jebb & Cheek (1997)	Schlauer (2000)	Clarke (1997) + (2001)	Cheek & Jebb (2001)	Kurata (2001)
N. adnata				O		O	O	O	O	
N. alata	O	O	O	O		O	O	O	O	
(N. alicae)	O	s				s	s	s	s	
(N. albolineata)	O	s				s	s	s	s	
N. albomarginata	O	O	O	O	O	O	O	O	O	
N. ampullaria	O	O	O	O	O	O	O	O	O	
N. anamensis	O	O	O			O	O			
(N. angasanensis)							s	O	s	
(N. angustifolia)	O	s				s	s	s	s	
N. argentii						O	O		O	
N. aristolochioides						O	O	O	O	
(N. armbrustae)	O	s				s	s		s	
(N. beccariana)	O	s				s	s	s	s	
N. bellii			O			O	O		O	
N. benstonei							O	O	O	
(N. bernaysii)	O	s				s	s	s	s	
N. bicalcarata	O	O	O		O	O	O	O	O	
(N. blancoi)	O	s				s	s		s	
N. bongso	O	O	O	O		O	O	O	O	
(N. borneensis)					O	O	s	s	s	
N. boschiana	O	O	O		O	O	O	O	O	
N. burbidgeae	O	O	O		O	O	O	O	O	
N. burkei	O	O	O			O	O		O	
N. campanulata			O		O	O	O	O	O	
(N. carunculata)		O	O	s		s	s	s	s	
(N. cholmondeleyi)	O	s				s	s	s	s	
N. clipeata		O	O		O	O	O	O	O	
(N. copelandii)	O	s				s	s		s	
N. danseri						O	O		O	
(N. deaniana)	O	O	O			*	O		*	
(N. decurrens)		O	O			s	s	s	s	
N. densiflora			O			O	O	O	O	
(N. dentata)			O		s	s			s	
N. diatas						O	O	O	O	
N. distillatoria	O	O	O			O	O			
N. dubia		O	O	s		O	O	O	O	
(N. echinostoma)	O	s				s	s	s	s	
N. edwardsiana	O	s	O		O	O	O	O	O	
N. ephippiata		O	O		O	O	O	O	O	
N. eustachya	O	s		s		O	O	O	O	
N. eymae						O	O		O	
N. faizaliana						s	O	O	O	
(N. falliax)	s	s				s	O	s	s	
N. fusca		O	O		O	O	O	O	O	
(N. garrawayae)	O	s				s	s	s	s	
(N. geoffrayi)		O	O			s	s		s	
(N. globamphora)			O			s	s		s	
N. glabrata						O	O		O	
N. gracilis	O	O	O	O	O	O	O	O	O	
N. gracillima	O	O	O			O	O	O	O	
N. gymnamphora		O	O			O	O	O	O	
N. hamata						O	O		O	
(N. hemsleyana)	O	s				s	s	s	s	
N. hirsuta	O	O	O		O	O	O	O	O	
N. hispida		s				O	s	O	O	
N. inermis		O	O	s		O	O	O	O	
N. insignis		O	O			O	O		O	
N. jacquelineae								O		
(N. jardinei)	O	s				s	s	s	s	
(N. kampotiana)		O	O			s	s		s	
(N. kennedyana)	O	s	O			s	s	s	s	
N. khasiana	O	O	O			O	O			
N. klossii		O	O			O	O		O	
N. lamii						O	O		O	
N. lavicola							O	O	O	
(N. leptochila)		O	O		O	s	s	s	s	

	Macfarlane (1908)	Danser (1928)	Kurata (1976)	Tamin & Hotta (1986)	Phillipps &Lamb (1996)	Jebb & Cheek (1997)	Schlauer (2000)	Clarke (1997) + (2001)	Cheek & Jebb (2001)	Kurata (2001)
N. longifolia						s	O	O	s	
N. lowii	O	O	O		O	O	O	O	O	
N. macfarlanei	O	O	O			O	O	O	O	
N. macrophylla					O	O	O	O	O	
N. macrovulgaris					O	O	O	O	O	
N. madagascariensis	O	O	O			O	O			
N. mapuluensis					O	O	O	O	O	
N. masoalensis						O	O			
N. maxima	O	O	O		O	O	O	O	O	
(N. melamphora)	O	s				s	s	s	s	
N. merrilliana		O	O			O	O		O	
N. mikei						O	O	O	O	
N. mindanaoensis										O
N. mira							O		O	
N. mirabilis		O	O	O	O	O	O	O	O	
N. mollis		O	O		O	O	O	O	O	
(N. moorei)	O	s				s	s	s	s	
N. muluensis			O		O	O	O	O	O	
N. murudensis					O	O	?	O	O	
(N. neglecta)	O	O	O			*	s	s	*	
N. neoguineensis		O	O			O	O		O	
N. northiana	O	O	O		O	O	O	O	O	
(N. oblanceolata)		O				s	s		s	
N. ovata						O	O	O	O	
N. paniculata		O	O			O	O		O	
N. papuana		O	O			O	O		O	
(N. pectinata)		O	O	s		O	s	s	O	
N. pervillei	O	O	O			O	O			
N. petiolata		O	O			O	O		O	
N. philippinensis	O	O				s	O		O	
N. pilosa		O	O		O	O	O	O	O	
(N. phyllamphora)	O	s		s		s	s	s	s	
N. rafflesiana	O	O	O	O	O	O	O	O	O	
N. rajah	O	O	O		O	O	O	O	O	
N. ramispina		s				O	s	O	O	
N. reinwardtiana	O	O	O	O	O	O	O	O	O	
N. rhombicaulis			O	O		O	O	O	O	
(N. rosulata)				O		s	s	s	s	
(N. rowenae)	O	s				s	s	s	s	
(N. sandakanensis)						s	s		s	
N. sanguinea	O	O	O			O	O	O	O	
N. sibuyanensis							O		O	
N. singalana	O	O	O	O	O	O	O	O	O	
(N. smilesii)	O	s				*	s		*	
N. spathulata			O	s		O	O	O	O	
N. spectabilis		O	O	O		O	O	O	O	
(N. spinosa)				O		s	s	s	s	
N. stenophylla	O	O	O		O	O	O	O	O	
N. sumatrana		s		O		O	O	O	O	
N. talangensis						s	O	O	s	
N. tentaculata	O	O	O		O	O	O	O	O	
N. tenuis						s	O	O	s	
N. thorelii		O	O			O	O			
N. tobaica		O	O	O		O	O	O	O	
N. tomoriana		O	O			O	O		O	
N. treubiana	O	O	O			O	O	O	O	
N. truncata		O	O			O	O		O	
(N. tubulosa)	O	s				s	s	s	s	
N. veitchii	O	O	O		O	O	O	O	O	
N. ventricosa	O	O	O			O	O		O	
N. vieillardii	O	O	O			O	O			
N. villosa	O	O	O		O	O	O	O	O	
(N. wilkiei)							s		s	
(N. xiphioides)						s	s	s	s	
TOTAL	(57)	(63)	(70)	(16) (スマトラ)	(32) (ボルネオ)	(82)	(88)	(62) (ボルネオ・スマトラ・マレー半島)	(79) (マレシア)	(1) (追加)

独立種（イタリック）＝91

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Purification and enzymatic characterization of an aspartic proteinase (Nepenthesin) from the insectivorous plant *Nepenthes distillatoria*

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Abstract. An aspartic proteinase (nepenthesin) was purified to apparent homogeneity, as examined by SDS-PAGE and NH_2-terminal sequencing, from the secretion of the insectivorous plant *Nepenthes distillatoria* by successive chromatographies on DEAE-cellulose, Sephacryl S-200, pepstatin-Sepharose and mono Q. The enzyme had a molecular mass of 45kDa as examined by SDS-PAGE and 58kDa by gel filtration, and was shown to be a glycoprotein. The NH_2-terminal 24-residue sequence was determined, which was quite different from those of other plant aspartic proteinases. It was optimally active at pH 3.0 to 3.5 and at 55°C toward hemoglobin. Below 50°C it was extremely stable over a wide range of pH for weeks where porcine pepsin is rapidly and irreversibly inactivated, indicating the evolutionary adaptation of the enzyme to its severe environment. Like porcine pepsin, the activity was strongly inhibited by aspartic proteinase-specific inhibotors: pepstatin and diazoacetyl-D,L-norleucine methyl ester in the presence of cupric ions. The B chain of oxidized insulin was cleaved mainly at the five peptide bonds, Phe24-Phe25, Glu13-Ala14, Leu6-Cya7, Leu15-Tyr16 and Tyr16-Leu17 by the enzyme. This cleavage specificity was similar, but not identical with those of any other aspartic proteinases reported, including pepsin and cathepsin D. These results indicate that nepenthesin is a unique aspartic proteinase in the aspartic proteinase family.

Introduction

Aspartic proteinases are widely distributed in living organisms and a number of studies have been performed on mammalian, fungal and viral aspartic proteinases (Barrett, 1995; Barrett *et al.*, 1998). They are also distributed widely in the plant kingdom, and are present in seeds, leaves and flowers in different plants as well as in

the digestive secretions of some insectivorous species (Kervinen *et al.*, 1995). Aspartic proteinases such as the phytepsin group (Kervinen, 1998), including barley (Runeberg-Roos *et al.*, 1991) and rice (Asakura *et al.*, 1995) aspartic proteinases and cyprosins (or cardosins) from the flowers of cardoon (Cordeiro *et al.*, 1995; Faro *et al.*, 1995; Cordeiro *et al.*, 1998; Pires, 1998a, b), have been well characterized. However, few studies were performed on proteinases of insectivorous plants. There are several different genera of plants that are insectivorous, which secrete acid proteinases to digest captured insects to obtain nitrogen (Darwin, 1875; Lloyd, 1942). These enzymes are interesting not only as targets for studies on structure-function relationships, but also from the standpoint of molecular evolution. Nepenthesin (Woessner, 1998) is the name given by Nakayama and Amagase (Nakayama and Amagase, 1968) to such an acid proteinase secreted in the pitcher of *Nepenthes* species. So far, acid proteinases from *Nepenthes* and *Drosera* species were partially purified and their properties investigated (Amagase *et al.*, 1969; Amagase, 1972; Jentsch, 1972; Lobareva *et al.*, 1973; Takahashi *et al.*, 1974; Tokes *et al.*, 1974). However, none of these enzymes were purified to homogeneity due to the difficulty to obtain a sufficient amount of their secretions. To overcome this problem, we collected a fairly large amount of the secretion from *Nepenthes distllatoria* (Badura), which grows abundantly in the tropical forests of Sri Lanka. This enabled us to purify the enzyme for the first time to homogeneity and to study various enzymatic characteristics using the purified enzyme. The results indicated that the enzyme outstands as a unique member of the aspartic proteinase family.

Materials and Methods

Materials: *Nepenthes* secretion was collected from the open pitchers of *Nepenthes distillatoria* at the forests of Sri Lanka. DEAE cellulose (DE-52) was a product of Whatman Co. Sephacryl S-200 and a Mono Q HR5/5 column were purchased from Pharmacia Biotechnology Inc. and pepstatin-Sepharose was from Sigma Chem. Co. The B chain of oxidized bovine insulin, diazoacetyl-D,L-norleucine methyl ester (DAN) and porcine pepsin were obtained from Sigma and pepstatin A was from Peptide Institute Inc. (Osaka, Japan). Reagents for automated amino acid analysis and sequencing were obtained from Applied Biosystems. Other reagents used were of the highest grade available.

Enzyme assay: Proteolytic activity of the enzyme was determined by the method of Anson (1939.) with modification (Athauda *et al.*, 1989.) at 37^{0}C using 2% denatured

hemoglobin in 0.1 M sodium formate buffer, pH 3.0, as a substrate. One unit of activity was defined as ΔA_{280nm}/h. The pH and temperature dependences of the enzyme activity were analyzed by assaying at different pH values and temperatures, respectively.

Protein determination: Protein was determined by measuring the absorbance at 280 nm of the sample solution or by the method of Smith *et al*. (1985) using the biscinchoninic acid reagent.

Purification procedures: The collected secretion (30 L) was filtered and dialyzed against 0.02 M sodium phosphate buffer, pH 7.5, (0.02 M in phosphate). The proteins in the dialyzed *Nepenthes* secretion were adsorbed to DEAE-cellulose gel by batch adsorption in the same buffer and the gel was placed in a column (6.0 x 70 cm). The column was washed with 0.02 M sodium phosphate buffer, pH 7.5, and the protein was eluted with the same buffer containing 0.5 M NaCl. The fractions with proteolytic activity were pooled and applied to a second DEAE-cellulose column (6.0 x 35 cm) equilibrated with 0.02 M sodium phosphate buffer, pH 7.5. The column was washed with the same buffer and the protein was eluted with a linear gradient of 0 to 0.5 M NaCl in 2 L of the same buffer. Fraction size, 10 mL. The active fractions (fraction nos. 51-82) were pooled and concentrated by using a small DEAE-cellulose column. The concentrated sample was applied to a Sephacryl S-200 column (3.1 x 114 cm) equilibrated with 0.02 M sodium phosphate buffer, pH 7.5, 0.2 M NaCl. Fraction size, 11 mL. The active fractions (fraction nos. 37-48) were pooled and dialyzed against 0.02 M sodium acetate buffer, pH 4.0, and applied to a pepstatin-Sepharose column (1.3 x 1.6 cm) equilibrated with the same buffer. The column was washed with 0.02 M Tris/HCl buffer, pH 8.0, containing 1 M NaCl, and the protein was eluted with the same buffer. The active fractions were pooled and dialyzed against 0.02 M Tris/HCl buffer, pH 7.8, and applied to a Mono Q column. The protein was eluted with a linear gradient of 0 to 1 M NaCl in 0.02 M Tris/HCl buffer, pH 7.8. The Mono Q chromatography was performed three times with one-third of the sample at a time, and the active fractions were pooled and combined.

Purity check and molecular weight determination: SDS-PAGE (Laemmli, 1970) and gel filtration on a column (3.1 x 114 cm) of Sephacryl S-200 were used

for purity check and molecular weight determination of the purified enzyme. In SDS-PAGE, the protein was stained with Coomassie Brilliant Blue and the carbohydrate with Schiff's reagent after periodate oxidation (Zacharius *et al.*, 1969.). The purity was also checked by NH_2-terminal amino acid sequence determination by using an Applied Biosystems pulse-liquid protein sequencer model 477A.

Reaction of diazoacetyl-D, L-norleucine methyl ester (DAN): The purified proteinase or porcine pepsin (50 μg) was treated with DAN (200mg) in 3.0 mL of 0.05 M sodium acetate buffer, pH 5.0, at 14 °C in the presence or absence of 2 mM cupric sulfate.

Stability studies: The enzyme solution (10 μg/200 μL of 0.05M Tris/HCl buffer, pH 8.0) was incubated at various temperatures for 1 h and then assay was performed under the standard conditions.

In order to investigate the effect of temperature on prolonged incubation of the enzyme, the enzyme (50 μg/mL of 0.02M sodium formate buffer, pH 3.0) was incubated at different temperatures (4, 25, 37 and 50 °C) for a period of 1 month. Porcine pepsin solution (50 μg/mL of 0.02M sodium formate buffer, pH 3.0) was incubated in the same way as a control. To investigate the effect of pH on prolonged incubation, the enzyme (50 μg/mL of buffer) was incubated at 37 °C for a period of 1 month. The bufferes used were 0.05M sodium formate buffer, pH 3.0, 0.05 M sodium acetate buffers, pH 4.0 and 5.0, 0.05 M sodium phosphate buffers, pH 6.0 and 7.0, 0.05 M Tris/HCl buffers, pH 8.0 and 9.0 and 0.05M sodium borate buffer, pH 10.0. Porcine pepsin solutions (50 μg/mL of respective buffers) were incubated in the same way. In both experiments, aliquots were removed at different time intervals (0 time, 1 day, 3 days, 7 days, 14 days, 21 days and 30 days) and the remaining activity was determined.

Digestion of oxidized insulin B chain and analysis of the cleavage sites: The B chain of oxidized insulin (150 nmol) was digested with the purified proteinase (0.3 nmol) in 600 μL of 0.1 M sodium formate buffer, pH 3.0, at 37°C for 3 h. The resulting peptides were separated by HPLC in the same manner as described above. An aliquot of each peptide fraction dissolved in water was submitted to amino acid analysis using an Applied Biosystems automated derivatizer

analyzer 420A/130A.

Results and Discussion

Upon the second DEAE-cellulose chromatography of the enzyme sample from the *Nepenthes* secretion, two acid proteinase activity peaks were obtained; the major peak (fraction nos. 51-82) was eluted at 0.25 M NaCl and the minor peak (fraction nos. 90-122) at 0.42 M NaCl. The subsequent studies were made on the major enzyme. The major enzyme was purified to homogeneity as examined by SDS-PAGE by successive chromatographies on columns of Sephacryl S-200, pepstatin-Sepharose and Mono Q with a 200-fold purification in a yield of 21%. This is the first case of complete purification of the enzyme. About 2mg of the acid proteinase was obtained from 30 L of secretion from the open pitchers of *Nepenthes*. Purification was also performed with the secretion (1.2 L) collected from the closed pitchers by the same procedure on a smaller scale; essentially the same results as above were obtained.

The molecular mass of the purified proteinase was estimated to be 45 kDa by SDS-PAGE under non-reducing conditions and 58 kDa by gel filtration. The protein band obtained by SDS-PAGE was stained with the periodic acid-Schiff's base reagent, suggesting that the enzyme is a glycoprotein. The NH_2-terminal amino acid sequencing gave a single 24-residue sequence: Ile-Gly-Pro-Ser-Gly-Val-Glu-Thr-Thr-Val-Tyr-Ala-Gly-Asp-Gly-Glu-Tyr-Leu-Met-Asn-Leu-Ser-Ile-Gly-.

Interestingly, this sequence has little homology with the NH_2-terminal sequences of barley (Runeberg-Roos *et al.*, 1991), rice (Asakura *et al.*, 1995) and cardoon flower (Cordeiro *et al.*, 1995.), suggesting an evolutionally distant relationship.

Like porcine pepsin, the enzyme was strongly inhibited by pepstatin, but none of the inhibitors specific for serine, cysteine and metallo proteinases were inhibitory, and complete inhibition was obtained at 0.1 mM pepstatin. Pepstatin was shown to bind to the enzyme in an approximately 1:1 stoichiometry. The enzyme was also inactivated by DAN in the presence of cupric ions in the same manner as porcine pepsin. Under the conditions used, the enzyme and porcine pepsin were inactivated almost completely in 3 h in the presence of cupric ions, and they were not inactivated at all in the absence of cupric ions. Since pepstatin and DAN are typical inhibitors specific for aspartic proteinases, these results clearly show that the enzyme belongs to the typical aspartic proteinase family.

The optimal activity of the enzyme toward denatured hemoglobin was observed at pH 3.0-3.5, which is appreciably higher than that of pepsin (~pH 2.0) and is rather similar to that of cathepsin D. The enzyme had some activity even above pH 6.0, where pepsin is inactive. The optimal temperature was 55 °C and the enzyme was stable up to this temperature when incubated at pH 8.0 for 1 h. Below 50 °C the

enzyme was extraordinarily stable in a wide range of pH (pH 3 to pH 10) for weeks, where porcine pepsin was rapidly and irreversibly inactivated. This unusual stability has never, to our knowledge, been reported for other proteinases, indicating an evolutionary adaptation of the enzyme to its severe environment.

Cleavage specificity of the enzyme was investigated by using the B chain of oxidized insulin as the substrate. Several peptide bonds were cleaved and especially the peptide bonds, Phe24-Phe25, Glu13-Ala14, Leu6-Cya7 (Cya, cysteic acid), Leu15-Tyr16 and Tyr16-Leu17, were cleaved to marked extents. The extents of cleavage of these bonds were estimated to be 80%, 55%, 50%, 38% and 35%, respectively, under the conditions used. Previously, the cleavage specificity of partially purified nepenthesin was investigated using various peptides (Amagase *et al.*, 1969) and ribonuclease (Tokes *et al.*, 1974.) as substrates. These results seem to coincide partially with the present results, although strict comparison is difficult due to the difference in the substrates used. The specificity of the enzyme appears to be similar, but not identical to those of other aspartic proteinases such as pepsin and cathepsin D (Barrett, 1977; Matsuzaki and Takahashi, 1988; Keil, 1992). Most peptide bonds susceptible to these enzymes were more or less cleaved by the enzyme. However, there is a remarkable difference: the Leu6-Cya7 bond was found to be one of the major sites of cleavage by nepenthesin whereas the cleavage of this bond has not been reported for other aspartic proteinases with a possible exception of porcine spleen cathepsin D which was reported to cleave this bond very slowly (Cunningham *et al.*, 1976; Keil, 1992). Leucine residue may be one of the preferred P1 site of the enzyme since three of the four Leu-X bonds in oxidized insulin B chain were more or less cleaved.

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Poster Abstracts and Papers

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A-1

Base specific double-staining method for chromosome identification in *Drosera filiformis* Raf.

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Abstract. The chromosomes of two strains of *Drosera filiformis* Raf. were analyzed in detail using DNA base specific chromosome banding technique. To develop chromosome banding technique, sequentially fluorescent staining with distamycin A (DMA) and chromomycin A_3 (CMA), sequentially fluorescent staining with actinomycin D (AMD) and 4'-6-diamidino-2-phenylindole (DAPI), and double staining with CMA and DAPI were employed. With sequentially fluorescent staining, *D. filiformis* showed CMA-negative and DAPI-positive bands on the mitotic chromosomes. In contrast, CMA-DAPI double staining method with dual-bandpath filter made two-colored chromosome image enhanced DNA base specific sequences. The double staining method had an advantage for a simultaneous detection to distinguish between AT rich hetelochromatin segments and other euchromatin segments in *D. filiformis*.

Introduction

Drosera filiformis Raf. had the largest chromosome size in the Northern Hemisphere species, and this is only one species to exhibit remarkable bands in each chromosome by DNA base specific fluorescent staining (Hoshi and Kondo, 1998). These data imply that *D. filiformis* is the most possible cantidate to identify chromosmoes in genus *Drosera*. However, according to the chromosome banding results in Hoshi and Kondo (1998, 1998a), more detail banding analysis and development of fluorescent staining technique should be necessary for chromosome identification and for further chromosomal phylogenic study in the genus.

Physical mapping and *in situ* hybridization has been performed on chromosomes in many plants to clarify chromosomal differentiation and evolution in related species. The physical mapping depends on a accurate cytological analysis and chromosome identification with stable bands (Daga *et al.*, 1996). Generally, the chromosomes have to be characterized using size, shape, position of primary constriction (centromere) and secondary constriction (nucleolar organizer region), and banding pattern. However, all *Drosera* chromosomes at mitotic chromosomes could not be classified by the method using localized-centromeric position, since they do not shown clearly localized primary constriction. Thus, it was very difficult to identify individual chromosomes using the standard karyotyping system. Because of the obscure constriction, a hypothesis of diffuse-centromeric chromosome have been proposed (Kondo and Segawa, 1988).

In this paper, two strains of *D. filiformis* were used and analyzed in detail by morphological and cytological methods, using DNA base specific chromosome banding technique. This study is the first attempt for chromosome identification to propose cytological map in carnivorous plants.

Materials and Methods

Plant materials Seeds of two strains of *Drosera filiformis* were purchased from Western Australia, Australia. They were surface-sterilized and sown on sterilized 1/2 Murashige and Skoog (MS) basal medium (Murashige and Skoog 1962) supplemented with 0.2 % gellan gum without any growth substance.

Slide preparation for fluorescent staining To observe mitotic chromosomes, root tips were collected and pretreated with 0.002 M hydroxyquinoline for two hours at 18 ∞C before fixation with 45 % acetic acid for five minutes. Then, these were hydrolyzed in a mixture of 1N hydrochloric acid and 45% acetic acid (2:1) at 60 ∞C for seven seconds. Then, they were squashed in 45 % acetic acid. The preparations were air-dried for 24 hours at room temperature after removal of coverslips with dry ice.

Sequentially fluorescent staining with distamycin A (DMA) and chromomycin A_3 (CMA) The method of Hoshi and Kondo (1998) was followed with slight modifications. Removing coverslips the air-dried slides were used for sequential DMA-CMA staining: The slides were preincubated in McIlvine's buffer (pH 7.0) for 30 minutes, treated with 0.1 mg/ml DMA (Sigma) in McIlvine's buffer for ten minutes in a humid chamber and rinsed in McIlvine's buffer supplemented with 2.5 mg/ml $MgSO_4$ for ten minutes. After rinsed, they were stained with 0.1 mg/ml CMA (Sigma) in McIlvine's buffer supplemented with $MgSO_4$ for ten minutes. Then, they were mounted with glycerol and stored at 4 ∞C for 12 hours to prevent fading. These chromosomes stained with DMA-CMA were irradiated with BV (blue violet) filter casette and fluoresced yellow.

Sequentially fluorescent staining with actinomycin D (AMD) and 4'-6-diamidino-2-phenylindole (DAPI) The methods of Hoshi and Kondo (1998) was followed with slight modifications. After DMA-CMA staining, the slides were used for sequential AMD-DAPI staining: The slides were destained in 45 % acetic acid for 30 minutes, rinsed in distilled water for five minutes. They were dipped in McIlvine's buffer for 30 minutes, treated in 0.25 mg/ml AMD (Sigma) in McIlvine's buffer for 15 minutes in a humid chamber and rinsed in McIlvine's buffer for ten minutes. After rinsed, they were stained with 0.1 μ g/ml DAPI (Sigma) in McIlvine's buffer for ten minutes. Then, they were mounted in glycerine and observed immediately. These chromosomes stained with AMD-DAPI were irradiated with UV (ultra violet) filter cassette and fluoresced blue. Photographs were taken the Professional T-MAX films (ISO 400) in a Nikon fluorescent microscope.

Double staining with CMA and DAPI For simultaneous detection of the chromosome segments enhanced adenin-thymine (AT) and guanine-cytosine (GC) rich sequences, dual-bandpath filter were used to observe the chromosomes stained with CMA and DAPI. Two-color images were taken by CoolSNAP camera on a Olympus BX40 fluorescent microscope.

Results and Discussion

Mitotic interphase nuclei *Drosera filiformis* displayed eight to 17 big CMA-negative and DAPI-positive blocks (1.0-2.0 μ m diameter) in the interphase nucleus. Among the species in sect. Rossolis, ser. Eurossolis, the CMA-negative and DAPI-positive heterochromatin blocks were shown in *D. intermedia* and *D.* x *hybrida* (the hybrid of *D. filiformis* and *D. intermedia*) (Hoshi and Kondo, 1998). Other species in same group did not show any block characterized by CMA-negative and DAPI-positive pattern (Hoshi and Kondo, 1998). With respect to this phenomenon, *D. filiformis* might be more closely

related to *D. intermedia* than to other *Drosera* species.

Prophase and prometaphase chromosomes In mitotic prophase and prometaphase chromosome, *Drosera filiformis* exhibited karyomorphologically the gradient type (Fig. 1). Hoshi and Kondo (1998) mentioned that the gradient type was observed in all species of sect. Rossolis, ser. Eurossolis. Thus, the graduent type might be a common feature in this series.

No localized-centromiric chromosome was observed. Two satellites were detected as CMA-positive and DAPI-negative dots, and were away from the satellite chromosomes. All chromosomes exhibited clearly CMA-negative and DAPI-positive bands. Similar chromosome banding pattern were reported in *D. intermedia* (Hoshi and Kondo, 1998). As well as the interphase result, prophase and prometaphase fluorescent banding analyses supported the close rerationship to *D. intermedia.* Moreover, the CMA-negative and DAPI-positive bands in *D. filiformis* made it possible to identify chromosomes, especially at prometaphase.

Metaphase chromosomes Clear and easy counts of chromosome number were possible in mitotic metaphase stage. Two strains were used in this study and showed different chromosome number (2n=20 and 2n=21). In this species, the strain with 2n=21 is the first report as trisomic aneuploid. No localized-centromeric chromosome was observed at metaphase in this species. Two chromosomes had the satellites which appeared as the characteristics of CMA-positive and DAPI-negative sites. Twenty CMA-negative and DAPI-positive bands appeared at the distal and the interstitial regions in the metaphase chromosomes of *D. filiformis.* In the previous chromosome banding analysis, *D. intermedia* did not show any clear fluorescent band at mitotic metaphase, but exhibited CMA-negative and DAPI-positive bands at prophase (Hoshi and Kondo, 1998). Chromomycin A_3 binds specifically to guanine regions in helical DNA (Ward *et al.*, 1965) or heterochromatin (Deumling, 1981; Deumling and Greilhuber, 1982), while DAPI binds specifically to AT base pairs in the minor groove of DNA (Portugal and Waring, 1988). The more stable metaphase DAPI-positive bands in *D. filiformis* could be explained by high content of AT base pairs in the heterochromatin regions.

The IBAS analysis were carried out to characterize *Drosera filiformis* chromosomes. *Drosera filiformis* showed the total chromosome area of more than 88 μm^2, average chromosome area of 4.4 μm^2. According to Hoshi and Kondo (1998), these values of chromosome analysis were the largest in all diploid species with basic chromosome number of x=10. Moreover, Kondo and Segawa (1988) described that *D. filiformis* had a series of the largest chromosomes at mitotic metaphase in six species in the Northern Hemisphere. The chromosome measurements in *D. filiformis* was the same as those of Kondo and Segawa (1988). Sumner (1990) described mechanisms to explain possible appearance of heterochromatic band as follows: New bands could form by process of euchromatin transformation and/or by amplification of an existing DNA sequence. According to this explanation, large chromosomes of *D. filiformis* seem to be due to amplification of AT-rich DNA sequence. Thus, *D. filiformis* might have advanced character in chromosome differentiation.

Chromosome identification Double staining method with dual-bandpath filter made two-colored chromosome image enhanced base specific DNA sequences. The method is good for a simultaneous detection to distinguish between AT rich hetelochromatin segments and other euchromatin segments in *D. filiformis.* Simultaneous detection brought big advantage to determine the accurate position of AT rich heterochromatin in *D. filiformis* chromosomes. This determination was quite difficult in sequential fluorescent banding method. After detail observing the chromosomes at pro- prometa- meta- and midmetaphase stages, we will suggested that the mitotic prometaphase was the best stage

for chromosome identification in this species. To propose high-quarity cytological map of *D. filiformis*, further investigation is expected using computer-aided imaging analysis with prometaphase chromosome banding pattern.

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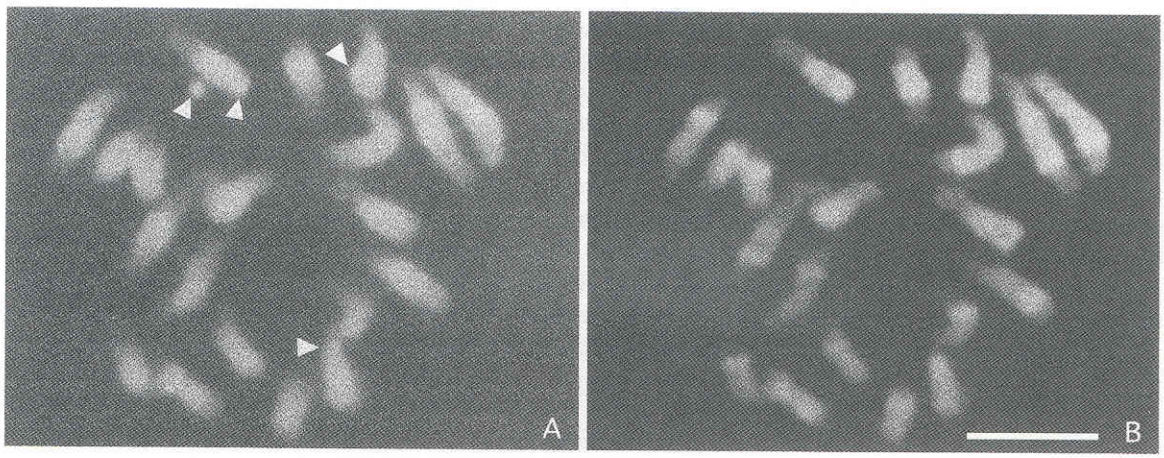

Figure 1. Mitotic prometaphase chromosomes in somatic cells of *Drosera filiformis* (2n=20) stained with DMA-CMA (A) and AMD-DAPI (B). CMA-positive and DAPI-negative sites (arrowheads) and CMA-negative and DAPI-positive bands were observed in this species.
Bar=10 μm.

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A molecular cytogenetic study in *Drosera filiformis* and *D. graminifolia*

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Abstract. Relationships between *Drosera filiformis* distributed in the South-eastern United States and *D. graminifolia* distributed in the Eastern Brazil were studied and compared by molecular cytogenetics using fluorescence *in situ* hybridization (FISH) with the probes of 45S ribosomal DNA (rDNA; by pTa71) and the *Arabidopsis*-type telomere sequence repeats. The two species had quite different orcein-stained karyotypes as well as chromosome numbers and exhibited 45S rDNA signals commonly at the terminal region of chromosomes but different signal numbers that showed no correlation. FISH using the *Arabidopsis*-type telomere sequence repeats indicated multi-minute signals occurred lineally on the chromosome along the gap between the sister chromatids as well as the both telomere regions of chromosome. Additionally, the chromosomes of *D. filiformis* showed another signals of the *Arabidopsis*-type telomere sequence repeats on the interstitial region and other regions *at random*. *Drosera filiformis* and *D. graminifolia* could be originated from different source and direction within the genus and are not closely related to each other.

Introduction

Among the members of the Droseraceae, *Drosera* is the largest genus and consists of somewhat 90～140 species (*e.g.*, Diels, 1906; Schlauer, 1996). A half of the species of the genus, ca 65 species have shown their chromosome numbers and karyotypes (*e.g.*, Hoshi and Kondo, 1998a, b). Kondo and his group (*e.g.*, Kondo *et al.*, 1976; Kondo and Lavarack, 1984; and so on in Kondo's early references) have discovered that *Drosera* chromosomes have diffused-centromeric habits. This was very first finding and only a case in the dicot plants. The series of our chromosomes researches in *Drosera* have been approving this peculiar phenomenon; light microscopic observation always showed no constrictionon the chromosomes observed (a lot of papers); γ-ray exposure produced a few to numerous fragments of chromosomes which were all survived since they supposedly had own centromeres (Sheikh *et al.*, 1995; Furuta and Kondo, 2000); Centromere banding technique always showed diffused-centromeric habits (Sheikh *et al.*, 1995), scanning electron microscopic observation showed masses of microtubules or spindle fibers and no primary constriction (Nontachaiyapoom *et al.*, 2000). Beside *Drosera* chromosomes, Aldrovanda chromosomes were speculated to have diffused centromeric chromosomes however, it was rather difficult to detect details in diffused-centromeric habit in *Aldrovand* chromosomes because of their too small chromosomes (Hoshi and Kondo, 1998a).

Chromosome sizes in *Drosera* are correlated with distribution locations of species (Hoshi and Kondo, 1998a, b). Australian *Drosera* shows well chromosomal diversity as well as morphological diversity but the *Drosera* species endemic to South Africa always show simply and commonly 2n=40 small chromosomes (Kondo and Oliver, 1979; Hoshi and Kondo, 1998; Nicholas and Kondo, 1998). However, chromosome observation in South American species of *Drosera* is very much lacking in most standard references.

Gene mapping on chromosome by FISH promises chromosome identification, analysis of chromosome structures, species relationships, and so on.

Materials and Methods

Drosera filiformis, D. graminifolia (Fig. 1) and their close relatives; total seven species used in this study are tabulated in Table 1. The reasons why *D. filiformis* and *D. graminifolia* were chosen for the main materials in this study are that (1) both species have quite similar leaf morphology of filiform, (2) different, allopatric distributions, and (3) different sizes of chromosomes.

They were all axenic-cultured in vitro on 1/2 strength of Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) supplemented with 1.5% sucrose, 0.3% Gelrite and no growth substance at pH 5.5 under environment at 23℃ under 1200 lux light intensity for 24 hours illumination.

The root tips harvested were pretreated in 0.002M 8-hydroxyquinoline at 18℃ for 2.5 hours and then, were fixed in ethanol-acetic acid (3:1) and stored at -4℃. They were treated with an ethanol series (70%, 35% and then, distilled water) for ten minutes each. Then, they were enzymatic-macerated in 3% cellulase Onozuka R10 (Yakult), 1.12% pectolyase Y-23 (Seishin) in the solution of 0.5 M EDTA and 2XSSC (1XSSC: 0.15 M NaCl+0.015M sodium citrate) at pH 4.5 at 37℃ for ca 30 minutes. After rinsed in distilled water for ten minutes, they were sealed with 45% acetic acid. The slides were placed in a freezer at -80℃ for five minutes or more and their cover-slips were removed for air-drying.

For orcein-staining, naked slides with preparations were added a few drops of 3% acetic-acid and were placed in a chamber moistened with 45% acetic acid for staining for five minutes.

The 18S-5.8S-26S rDNA of wheat, pTa71, which contained the spacer region, was used as 45S rDNA probe (Gerlach and Bedbrook, 1979).

The *Arabidopsis*-type telomere sequence repeats was produced by the polymerase chain reaction (PCR) method. The PCR reaction mixture consisted of 0.2 M dNTP (TAKARA), 1.25 units Taq polymerase (TAKARA Taq; TAKARA), 0.5 mM each of the primers of 5'-TTTAGGG-3' and 5'-CCCTAAA-3' and 1XPCR buffer (TAKARA). Cycles of the PCR were described as follows: The first cycle was denaturation at 95℃ for 90 seconds, annealing at 55℃ for 30 seconds and primer extension 75℃ for 30 seconds. After DNA amplified oligonucleotides were confirmed by electrophoresis, they were precipitated by ethanol and resuspended in TE solution.

The 45S rDNA and the Arabidopsis-type telomere sequence repeats were labeled by nick translation (Bionick labeling System: Life Technologies, GIBCO BRL).

The FISH method used was of Laboratory of Plant Chromosome and Gene Stock, Graduate School of Science, Hiroshima University. The preparations were treated by 50 μl Rnase solution containing 0.1 mg/ml Rnase A (Sigma) in 2XSSC at 37℃ for one hour. After washing in 2XSSC for 15 minutes, they were refixed in 4% paraformaldehide dissolved in PBS at room temperature for ten minutes. The preparations were then washed in sterilized water and treated with an thanol series (70, 80, 100% five minutes each). After they were air-dried, 10 μl of the predenatured 45S rDNA or the *Arabidopsis*-type telomere sequence repeats probe mixture were applied to the slides and covered with silicone-coated cover-slips and sealed. The slides were denatured at 80℃ for three minutes and incubated at 37℃ overnight in Omnislide (HYBAID). They were washed in 2XSSC twice for ten minutes each. Then, they were dropped 70 μl of 1.5% bovine serum albumin (Sigma), 1.5% blocking reagent (Boehringer Mannheim) in 0.1 M Tris-HCl and 0.15 M NaCl at pH 7.0 and covered and sealed with a sealing film (Parafilm) for 15 minutes. The hybridization probes were detected by 20 μg/ml avidin-FITC conjugate (Sigma) and counterstained by 1 μg/ml propidium iodide (PI; Sigma). The slides were observed under fluorescence microscope using a blue filter (B-2A; Nikon). Microphotographs were taken on Super G400 (FUJI) film.

Since visualization of the signal of the *Arabidopsis*-type telomere sequence repeats had difficulty, signal amplification method was applied

Results and Discussion

Orcein-karyotyping The chromosome number of 2n=40 for *Drosera graminifolia* and that of 2n=20 for *D. sessilifolia* were recorded here for the first time. The chromosome number of 2n=20 found here was new to the South American species of *Drosera*. The chromosome numbers and sizes for the seven species of *Drosera* are tabulated in Table 3.

Centromeric regions in their chromosomes were not seen. Interphase chromosomes in all the species studied were commonly identified as "complex chromocenter type." The species in northern hemisphere always had single modality of chromosome sizes in their karyotypes at mitotic metaphase while *D. graminifolia* as well as *D. sessilifolia* had commonly bi-modality in their karyotypes consisted of two large chromosomes and the other similar almost the same sized chromosomes.

FISH using 45S rDNA The five species of *Drosera* studied commonly had 45S rDNA signals at the terminal region of chromosome (Figs. 2 and 3). The signal numbers found in the five species are tabulated in Table 4. This phenomenon supports Furuta and Kondo (1999); they observed the same phenomenon in *D. falconerii*. *Drosera graminifolia* (2n=40) and *D. sessilifolia* (2n=20) did not show any simple correlation with 45S rDNA signal numbers. *Drosera* of sect. Drosera commonly showed the 45S rDNA signals on certain middle-sized chromosomes, while *D. sessilifolia* of sect. Thelocalyx had the signals on larger-sized chromosomes (first, second, fifth and sixth chromosomes).

FISH using the *Arabidopsis*-type telomere sequence repeats Since this kind of signal in *Drosera* studied were so small and faint, signals were physically amplified by using secondary antibody method. Chromosomes of *D. filiformis* showed double dot signals of the *Arabidopsis*-type telomere sequence repeats near the terminal region, not exactly at the terminal region, and the interstitial region and furthermore chromosome region around the gap between the sister chromatids (Fig. 4). Chromosomes of *D. graminifolia* showed double dot signals at the both terminal regions of all of the chromosomes and single dot signals at the central region on the gap between the sister chromatids of 12 chromosomes (Fig. 4). According to Kondo and Furuta (1999) recorded signals of the *Arabidopsis*-type telomere sequence repeats in *D. falconerii* were dispersed throughout the chromosomes. On the other hand, diffused-centromeric chromosomes of monocot *Luzula luzuloides* showed the *Arabidopsis*-type telomere sequence repeats at the terminal region and sometimes at the interstitial region in order (Fuchs *et al.*, 1995). However, the present study showed chromosomes of *D. filiformis* and *D. graminifolia* had signals of the *Arabidopsis*-type telomere sequence repeats more or less in order. In case of *Allium cepa*, the terminal heterochromatic region of chromosomes could be covered by rDNA sequences or 375bp satellite DNA sequences to preserve chromosome terminal regions (Pich and Schubert, 1998). Drosera chromosomes with diffused centromeres may have some specific structures like *Allium cepa*. The signals in the chromosome of the Drosera species studied along the gap between the sister chromatids were seemed to be sometimes situated or conjugated in connection between sister chromatids.

As a conclusion, *D. filiformis* and *D. graminifolia* seemed to be originated from different strain and face to different evolutionary directions.

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Table 1. Materials used in this study

Species*	Distribution**	Source	Chromosome number (2n)	*in vitro* or *in vivo*
Subgen. Drosera				
Sect. Drosera L.				
Drosera brevifolia Pursh	Atlantic North America Brazil, Uruguay	Harsteal, U.S.A.	20	*in vitro*
D. capillaris Poir.	Atlantic North America Cuba,Guiana, Honduras		20	*in vitro*
D. filiformis Raf. var. *filiformis*	Atlantic North America	Nagoya, Japan cultiv.	20	*in vitro* and *in vivo*
D. graminifolia St. Hil.	Brazil, Guiana Venezuela	Caracas, Brazil	40	*in vitro* and *in vivo*
D. montana St. Hil.	Brazil, Venezuela	Itambe Peak, Brazil	40	*in vitro*
D. villosa St. Hil.	Brazil	Nagoya, Japan cultiv.	40	*in vitro*
Sect. Thelocalyx Planch.				
D. sessilifolia St. Hil.	Brazil, Guiana	U.S.A. cultiv.	20	*in vitro*

After *Seine and Barthlott (1994) and **Diels (1906)

Table 2. Differences between *Drosera filiformis* var. *filiformis* and *D. graminifolia*

	Leaves					
Species	similarities	differences	Stipules	Seeds	Flowers	Habitat
D. filiformis var. *filiformis*	threadlike leaves, red head glands on leaves,	up to 50cm long, glandular hairs with green stalks	fulvous lanuginose form, adnate basal leaves	ovoid and/or ellipsoid form, papillate testa	1.5cm across rose pink petal	peaty and sandy soil, savannah and margin of bogs
D. graminifolia	petioles indistinct, almost absent	over 30cm long, glandular hairs with red stalks	fuscuous membranous form, short acute dentate apex	narrowly fusiform	2cm across pink-lilac, red petal	rock and/or sand hills with wet surface

After Diels (1906), Schnell (1976) and Rivadavia (1996)

Table 3. Chromosome numbers and lengths of seven species of *Drosera* studied

Species	Chromosome number (2n)	Range of chromosome length (μm)
Drosera brevifolia	20	1.59-1.02
D. capillaris	20	2.48-1.55
D. filiformis var. *filiformis*	20	2.95-1.50
D. graminifolia	40	1.56-0.43
D. montana	40	0.93-0.45
D. villosa	40	1.09-0.56
D. sessilifolia	20	1.13-0.54

Table 4. Signal numbers in chromosomes of five species of *Drosera* studied by FISH using 45S rDNA probe

Species	Signal numbers		Chromosome number (2n)
	Interphase	Metaphase chromosomes	
Drosera brevifolia	1-7	2	20
D. capillaris	1-5	2	20
D. filiformis var. *filiformis*	1-6	4	20
D. graminifolia	1-8	6	40
D. sessilifolia	1-4	4	20

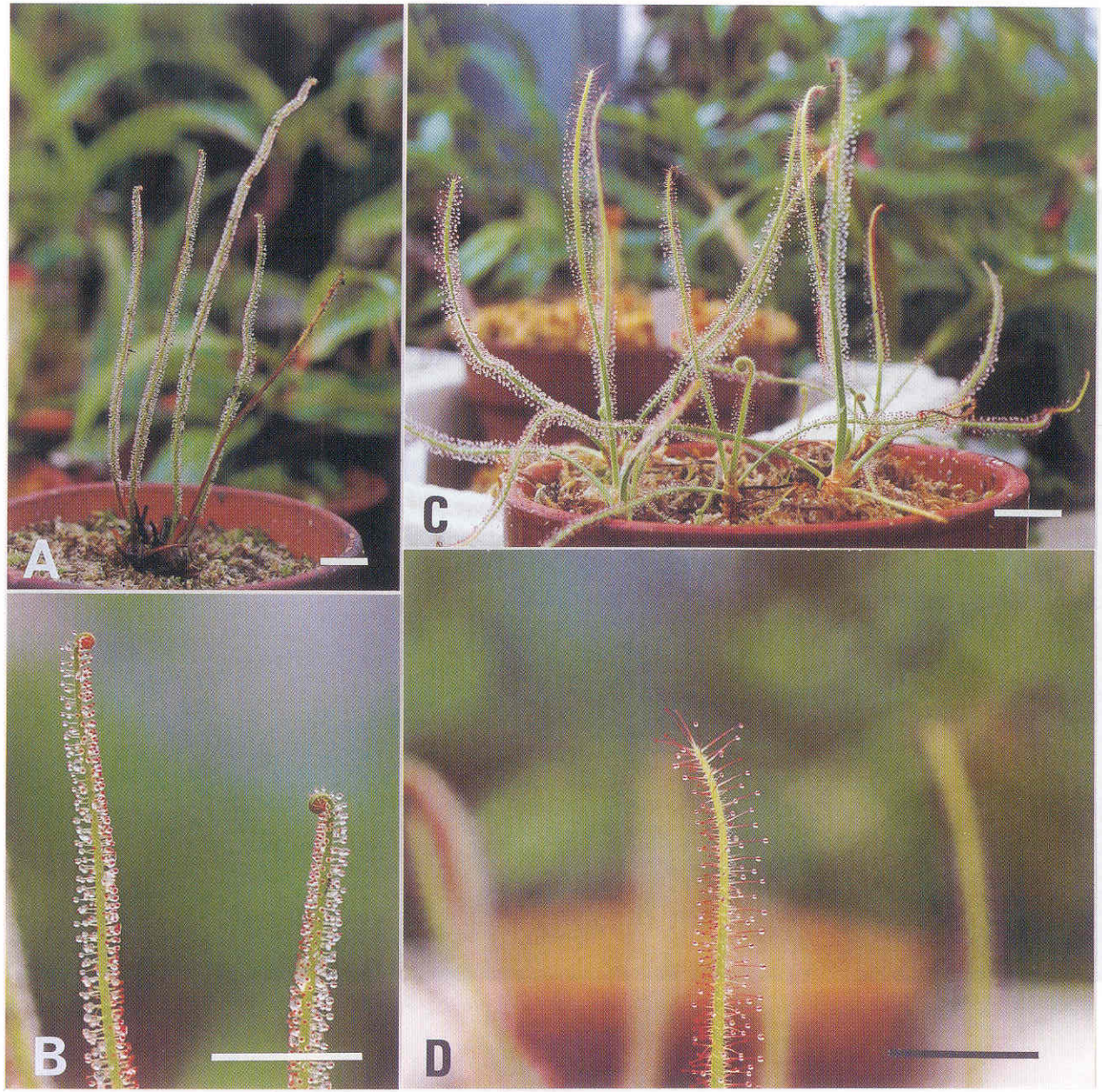

Figure 1. *Drosera filiformis* Raf. var. *filiformis* (A and B) and *D. graminifolia* St. Hil. (C and D)

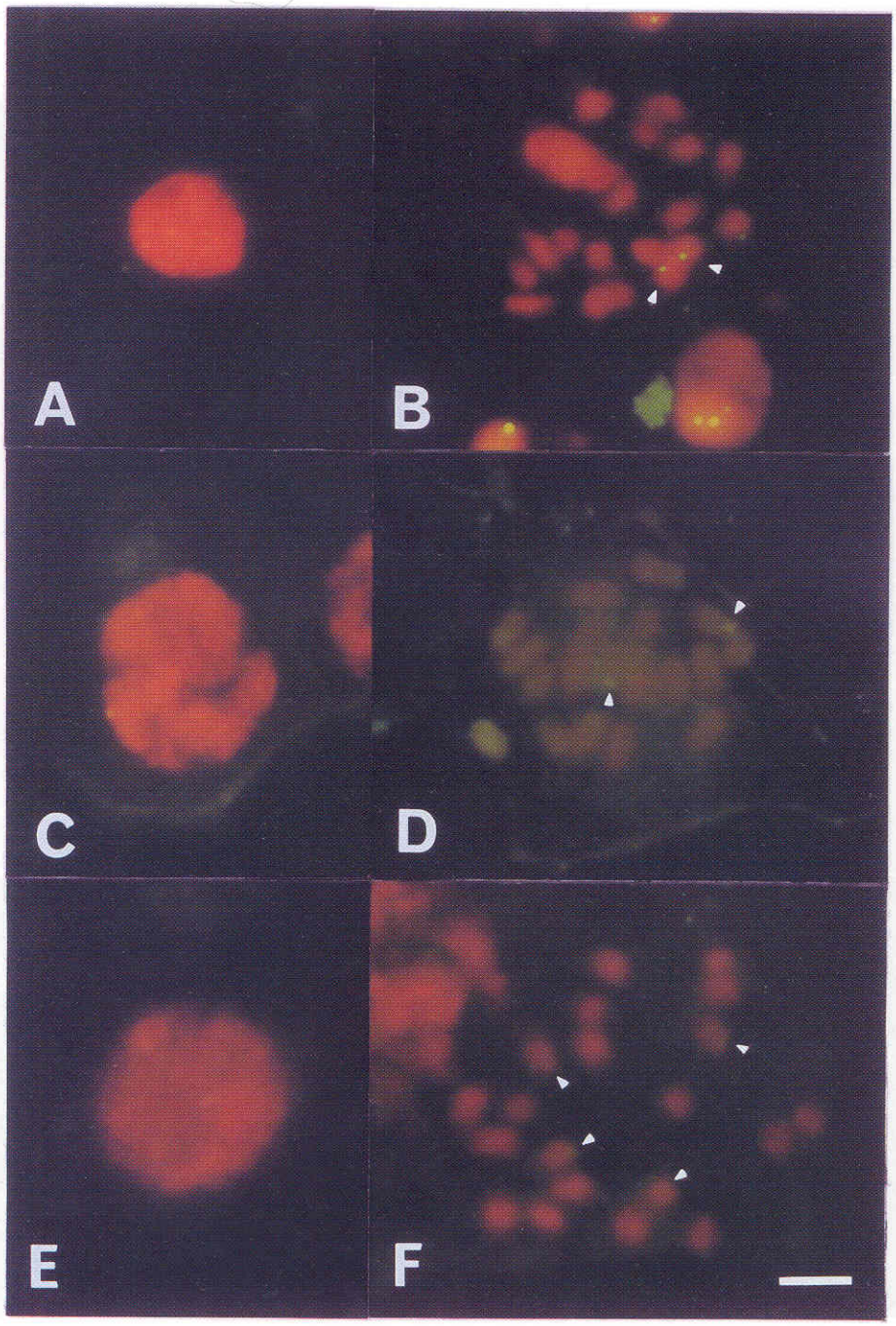

Figure 2. 45S rDNA signals by FISH in chromosomes of three species of *Drosera* in North America. A and B. *D. brevifolia*. C and D. *D. capillaris*. E and F. *D. filiformis* var *filiformis*. A, C, and E. Interphase chromosomes. B, D and F. Mitotic metaphase chromosomes. Bar=5 μ m.

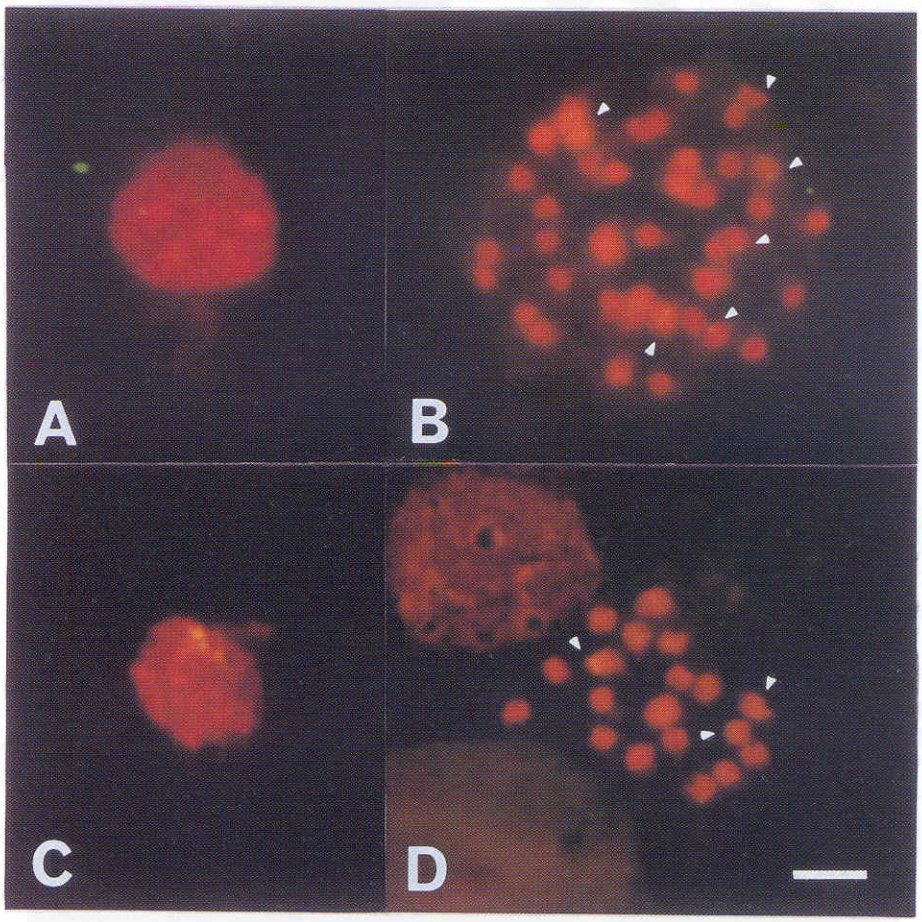

Figure 3. 45S rDNA signals by FISH in chromosomes of *Drosera graminifolia* (A and B) and *D. sessilifolia* (C and D) in South America. A and C. Interphase chromosomes. B and D. Mitotic metaphase chromosomes. Bar=5 μ m.

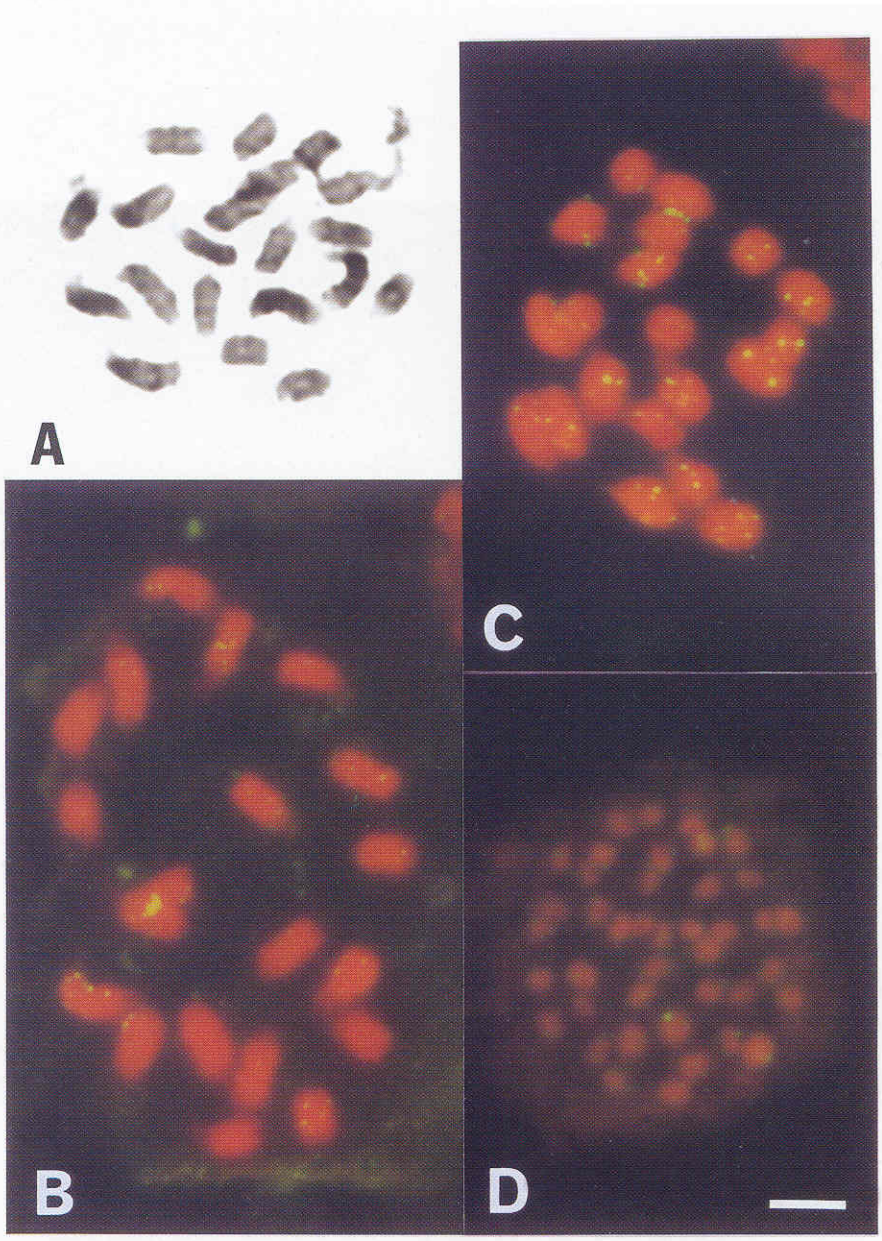

Figure 4. Diffused-centromeric natures of *Drosera* chromosomes analyzed by FISH using the *Arabidopsis*-type telomere sequence repeats. A. Orcein-stained chromosomes for comparison. B-D. Numerous small signals of the *Arabidopsis*-type telomere sequence repeats in mitotic mid-metaphase chromosomes. B and C. *D. filiformis* var. *filiformis*. D. *D. graminifolia*. Bar=5 μ m.

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Characterization of the nucleases secreted from *Drosera adelae*

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Abstract. Using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), we separated proteins involved in the digestive liquids of an insectivorous plant *Drosera adelae* from one another. They were recovered from the gel and assayed for nuclease activity. Several proteins could digest nucleic acids. One band of ca. 34 kDa showed a deoxyribonuclease activity. This enzyme acted as an endonuclease on double-stranded DNA and generated oligonucleotides with 3'-hydroxyl and 5'-phosphate ends. The activity of the enzyme was high in the range from pH 4.0 to 6.0. The enzyme required magnesium ions for its activity.

Introduction

Phosphodiester bonds in both DNA and RNA are extraordinary resistant to hydrolysis or nucleophilic attack. The enzymes that catalyze the cleavage of the phosphodiester bonds in nucleic acids are called nucleases. They have been the object of intensive study for many years and from many angles. However, to our knowledge, the study on the nucleases of insectivorous plants seems to be limited (Okabe and Ohyama, 1997).

The ability to trap and digest insects and other similar-sized animals has evolved in several plants that are called insectivorous or carnivorous plants (Juniper *et al.*, 1989). These plants usually grow in habitats poor in nitrates and ammonia. Therefore, in order to obtain the nitrogen they need for amino acids and nucleotides synthesis, they trap and digest insects. *Drosera* (sundew) has foliage leaves with elaborate glandular tentacles that secrete a sticky digestive liquid. The leaves usually curling up to enclose and digest caught insects. It is known that the digestive liquid contains proteases, phosphatases, nucleases, and so forth. However, detailed characterization of nucleases has not yet been carried out.

D. adelae, which grows in Australia, is one of the biggest sundews (Fig. 1). It has 5 to 10 leaves and each leave has 300~400 tentacles. The volume of the digestive liquid secreted from each tentacle is about 0.2 ml. Thus, we can easily obtain a great quantity of the digestive liquid from a single *D. adelae*. Using the liquid, we partially purified several nucleases using electrophoretic technique. Here, the nature of a deoxyribonuclease thus obtained is mainly described.

Materials and Methods

Electrophoretic separation of proteins Separation of proteins by SDS-PAGE was carried out according to Laemmli (1970) with slight modifications. The sample solution was prepared by mixing 9 ml of the digestive liquid and 3 ml of the solution containing 65 mM Tris-HCl (pH 6.8), 2% SDS, 5% dithiothreitol, 10% glycerol, and 0.002% BPB. After the incubation at 65°C for 15 min, it was loaded into three wells of the gel (4 ml per one lane) and electrophoresed. Side lanes were stained with silver salts after the electrophoresis. By reference to the signal positions in them, the gel pieces containing proteins were excised from the remainder center lane. Each gel piece was transferred into 100 ml of H_2O. Then, it was crushed there and the eluate of protein(s) was subjected to the digestion of DNA.

Digestion of DNA

In the initial experiment to detect nuclease activities, about 0.1 mg supercoiled plasmid DNA was mixed with each protein eluate, which was incubated at 30°C for 5 hr. The characterization

of a deoxyribonuclease thus identified was carried out as follows. The divalent-metal-cation requirement of the enzyme was investigated by the following procedure. At first, 3.5 ml of eluate and 0.5 ml of 50 mM EDTA were mixed and incubated at 30°C for 1 hr. Subsequently, 1 ml solution containing 0.1 mg supercoiled plasmid DNA and 50 mM $MgCl_2$ or $CaCl_2$ was added to the former solution. The reaction mixture was incubated at 30°C for 48 hr. The optimum pH for the enzyme was investigated using citrate buffer of various pH. Each of 5 ml reaction mixture contained 1 ml of the eluate of the deoxyribonuclease, 0.1 mg of supercoiled plasmid DNA and 20 mM citrate buffer and the reaction was carried out at 30°C for 5 hr. All reaction products were analyzed using agarose gel electrophoresis.

Results and Discussion

When supercoiled DNA molecules were digested with a small amount of the digestive liquid of *D. adelae*, nicked circular molecules and the linearized molecules were produced (not shown). This indicated that the liquid contained some endonuclease (or endonucleases). We intended to purify and characterize it (or them). The digestive liquid was subjected to SDS-PAGE and the proteins involved were separated from one another. They were recovered from the gel and subjected to the screening of nuclease activity. As the result, we identified several bands that showed this activity (not shown). Furthermore, it was found that a protein of ca. 34 kDa could digest supercoiled DNA molecules (Fig. 2A).

This enzyme was completely deprived of its activity in the presence of 5 mM EDTA (lane 3). The addition of $MgCl_2$ to the solution, however, resulted in the partial recovery of the activity (lane 4). While, the effect of $CaCl_2$ was very small (lane 5). These results indicated that magnesium ions were required for the activity of the enzyme. Concerning the mechanism of digestion, the following possibilities are raised. The result that linear DNA molecules of full length were generated in the process of digestion reminded us of DNase I, which cleaves both strands at approximately the same site in the presence of Mn^{2+} (Melgar and Goldthwait, 1968). The *D. adelae* deoxyribonuclease may have a similar mechanism of digestion. Alternatively, it may digest the closed circular DNA molecules in the following manner: i.e., (i) at first, it introduces nicks into closed circular DNA randomly; (ii) as the reaction proceeds, a coincidence of the sites of nicks between Watson and Crick strands increases, which generates not only linear DNA molecules of full length but also small DNA fragments (see lane 2). A study to know the molecular mechanism of the digestion is now in progress.

The resulting fragments were not phosphorylated with T4 polynucleotide kinase when they were not dephosphorylated precedently. While, dephosphorylated molecules were easily phosphorylated (not shown). These results strongly indicated that the enzyme generated oligonucleotides with 3'-hydroxyl and 5'-phosphate ends. The optimum pH for the enzyme activity was also investigated. It was revealed that the activity of the nuclease was high in the range from pH 4.0 to 6.0 (Fig. 2B).

The question which we must consider next is whether the deoxyribonuclease activity was derived from a single protein. Analysis of the amino acid sequence indicated that a single protein was responsible for the activity. Further work is in progress to clone cDNA of this deoxyribonuclease. Finally, we also report that we purified a ribonuclease that showed unique activity. Further characterization of the enzyme is now in progress as well.

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Figure 1. *Drosera adelae*

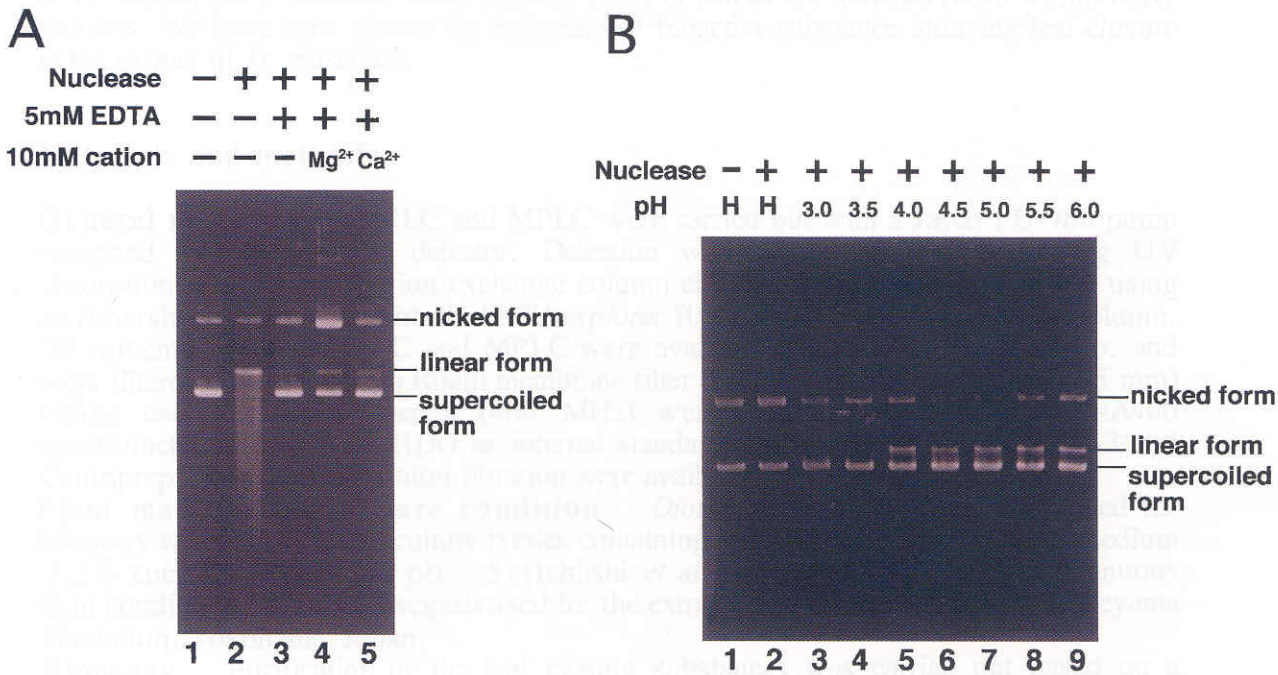

Figure 2. Divalent-metal-cation requirement (A) and optimum pH (B) of *D. adelae* deoxyribonuclease. In lanes 1 and 2 in panel B, H_2O was used instead of the citrate buffer, which is indicated with "H".

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Chemical Substances Inducing Leaf Closing Movement of *Dionaea muscipula* Ellis

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Abstract. We revealed that internal bioactive substance would be induced leaf closing of *Dionaea muscipula* Ellis without stimuli. The purification of this bioactive substance was examined according to the bioassay using the leaves of *D. muscipula*.

Introduction

Dionaea muscipula is the most famous carnivorous plant which C. Darwin claimed to be "the most amazing plant in the world" (Darwin, 1882). This plant can survive under the nutritionally poor soil condition by capturing insects with its leaves. The leaf closure of *D. muscipula* is induced when sensory hairs in leaves are touched twice within thirty seconds. We have now shown the existence of bioactive substance inducing leaf closure in the extract of *D. muscipula*.

Materials and methods

General notes The HPLC and MPLC were carried out with a Jasco PU-980 pump equipped with a UV-970 detector. Detection was carried out by monitoring UV absorption at 215 nm. The ion exchange column chromatography was carried out using an Amersham Pharmacia Biotech AKTA*explorer* 10S equipped with appropriate column. All solvents used for HPLC and MPLC were available from Kanto Chemical Co. and were filtered through a Toyo Roshi membrane filter (cellulose acetate, pore size 0.45 mm) before use. ^{1}H NMR spectra (400 MHz) were recorded on a Jeol JNM-A400 spectrometer in D_2O with HDO as internal standard (4.65 ppm). CentriprepYM-3 and Centriprep YM-10 used for ultra filtration were available from Millipore Co.

Plant material and culture condition *Dionaea muscipula* 'Red Giant' used for bioassay was grown in the culture bottles containing modified Murashige-Skoog medium (1.5% sucrose, 0.8% agar, pH 5.5) (Ichiishi *et al.*, 1999) at 27 ℃ under continuous light condition. *Dionaea muscipula* used for the extraction was purchased from Yoneyama Plantation, Yokohama, Japan.

Bioassay Purification of the leaf closing substances was carried out based on a bioassay using leaf of tissue cultured *D. muscipula.* The use of clone stain gave good reproducibility to the bioassay. The 3-month-old leaves were detached from the root with a razor blade. Each leaf was placed in a 5-mL glass vial containing distilled water (200 mL). After 24 hours, the distilled water was removed and each sample solution (100 mL) was added to each vial. The bioactive fraction made leaves closed after addition of a sample solution.

Extraction of bioactive substance for leaf closure Fresh trap leaves of *D. muscipula* (777 g) were extracted with 10% aqueous MeOH (4 L) for one week at 4 ℃

and centrifuged at 10000×*g* for 20 min at 4 ℃. The supernatant was concentrated to 1 L *in vacuo* and partitioned with *n*-hexane (500 mL×3), ethyl acetate (500 mL×3), *n*-butanol (500 mL×3). The aqueous layer was separated with Centriprep YM-10 at 2000 ×*g* for 90 min×4 at 4 ℃ and YM-3 at 2000×*g* for 90 min at 4 ℃, each process were repeated four times. The high-molecular weight fraction (M.W. 10000-3000) and low-molecular weight fraction (M.W. <3000) were obtained.

Purification of the high-molecular weight fraction The high-molecular weight fraction was purified with TOYOPEARL HW-40S column chromatography (*f*_50 ×500 mm, Tosoh Co.) with 20 % aqueous MeOH. Also this fraction was applied to Hiprep 16/10 Q XL column (Amersharm Pharmaia Biotech) equilibrated with 50 mM Tris-HCl Buffer (pH 8.0). The column was washed with 100 mL starting buffer at a flow rate of 4.0 mL/min, then eluted with 150 mL Tris-HCl Buffer (pH 8.0) containing 1 M NaCl. The bound fraction was desalted with Hiprep 16/10 Desalting column (Amersharm Pharmaia Biotech) with H_2O.

Purification of the low-molecular weight fraction The low-molecular weight fraction was purified by repeated TOYOPEARL HW-40S column chromatography (*f* 50×500 mm, Tosoh Co.) with 20 % aqueous MeOH (flow rate: 2.0 mL/min, detection: 215 nm). Further purification was carried out by HPLC using Develosil ODS-HG-5 column (*f* 20×250 mm, Nomura Chemicals Co.) with 5% aqueous MeOH (flow rate: 4.0 mL/min, detection: 215 nm).

Results and Discussion

The extract of *D. muscipula* induces leaf closure without stimulus. This result indicated that the leaf movement of *D. muscipula* would be induced by the internal bioactive substance. We tried to identify bioactive substance for leaf closure of this plant. Purification of the bioactive substance was carried out based on a bioassay using cultured *D. muscipula*. The bioactive substance was extracted from trap leaves with 10% aqueous MeOH and partitioned with *n*-hexane, ethyl acetate, *n*-butanol. The resulting aqueous layer showed weak leaf-closing activity. After ultra filtration of this aqueous layer, the high-molecular weight and the low-molecular weight fraction showed leaf closing activity at 20 g/L and 10 g/L, respectively.

The high-molecular weight fraction purified with gel filtration, the resulting bioactive fraction induced the leaf closing at 5g/L. In addition, ^{1}H NMR spectrum of this fraction indicated that main component of this fraction was a polysaccharide. Also this fraction was purified with anion exchange chromatography then the desalted bound fraction showed activity at 10 g/L.

After repeated gel filtration of the low-molecular weight fraction, the bioactive fraction showed strong leaf closing activity at 10g/L. Further purification was carried out with HPLC using Develosil ODS-HG-5 column and obtained bioactive fraction shows leaf closing activity at 5g/L.

These results suggest that an acidic polysaccharide and low molecular weight substance play important roles in the leaf closure of *D. muscipula*. Investigation on the separation condition for further purification of two bioactive substances for leaf closure is now in progress.

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Optimization of medium for growing the aquatic carnivorous plant *Aldrovanda vesiculosa in vitro*

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Abstract. The effect of KNO_3 and N-containing organic substances in Gamborg's B5 liquid medium on the growth of a rare aquatic carnivorous plant *Aldrovanda vesiculosa* was investigated in an aseptic culture *in vitro*. In Polish plants growing in a half-strength B5 medium with different KNO_3 concentrations, the medium with 1000 mg.l^{-1} KNO_3 was the best in all characteristics. The addition of 100-1000 mg.l^{-1} peptone to half-strength B5 medium with 500 mg.l^{-1} KNO_3 for Polish plants led to a step-wise increase of all growth characteristics but usually only the effect of 300 and 1000 mg.l^{-1} peptone was statistically significantly different from the absence of peptone. No clear relationship was found between growth characteristics and KNO_3 concentration in a half-strength B5 for Japanese plants. The addition of 100-1000 mg.l^{-1} peptone to a half-strength B5 medium with 500 mg.l^{-1} KNO_3 led to a two- to threefold increase in the number of shoot apices. pH values below 3.0 in used B5 media were not toxic for the growth of *Aldrovanda in vitro*. In both strains of *Aldrovanda* tested, the full-strength B5 medium with 2500 mg.l^{-1} KNO_3 appeared to be excessively concentrated for optimal growth.

Abbreviations. KNO_3, potassium nitrate; $(NH_4)_2SO_4$, ammonium sulfate; NaH_2PO_4, sodium phosphate; $CaCl_2 \cdot 6H_2O$, ; calcium chloride hexahydrate; $MgSO_4 \cdot 7H_2O$, magnesium sulfate heptahydrate; $FeSO_4 \cdot 7H_2O$, iron sulphate heptahydrate; Na_2EDTA, sodium ethylenediaminetetraacetic acid; H_3BO_3, boric acid; $MnSO_4$, manganese sulfate; $ZnSO_4 \cdot 7H_2O$, zinc sulfate heptahydrate; $CuSO_4 \cdot 5H_2O$, copper sulfate pentahydrate; $CoCl_2 \cdot 6H_2O$, cobalt sulfate hexahydrate; $Na_2MoO_4 \cdot 2H_2O$, sodium molybdate dihydrate; KI, potassium iodide; SE, standard error; N, nitrate; K, potassium.

Aldrovanda vesiculosa L. (Droseraceae) is a critically endangered and rare aquatic carnivorous plant of the Old World. It is rootless, free-floating, and grows just below the surface in shallow standing dystrophic waters (Lloyd, 1942; Adamec, 1995). For conservation and study, Kondo *et al.* (1997) developed a method of growing of *Aldrovanda* strains from Japan and E Poland in a sterile *in vitro* culture. They used Gamborg's B5 liquid medium (B5; Gamborg *et al.*, 1968) with 2% sucrose and 2500 mg.l^{-1} KNO_3. Since this medium is rather concentrated, Adamec and Pásek (2000) tried to modify it and preliminarily found a half-strength B5 medium with only 500 mg.l^{-1} KNO_3 to be the best for *Aldrovanda* growth and branching of shoots. However, pH values in all exhausted B5 media were very low, from 2.94 to 3.41. These pH values are far the optimum for the growth of *Aldrovanda*, which was found to be about 4.5 in a rather diluted mineral nutrient solution (Kaminski, 1987) or about 6.5 at natural sites (Adamec, 1997a). The acidification of B5 media could be caused by the fact that *Aldrovanda* greatly prefers the uptake of NH_4^+ to NO_3^- (Adamec, 2000). It is well-known that NH_4^+ uptake results in strong medium acidification, while NO_3^- uptake to alkalization (e.g., Marschner, 1995). Moreover, aquatic carnivorous plants can take up a great deal of their total N gain in the form of organic substances (for a review see Adamec, 1997b).

In this paper, we have investigated the effect of KNO_3 and N-containing organic substances in B5 medium on the growth of *A. vesiculosa* in an aseptic culture *in vitro*.

Aldrovanda plants from E Poland (Lake Dlugie) and Japan (Hozoji Pond, Hanyu City; Kondo *et al.*, 1997) used for the experiments were pre-cultured in a sterile culture in one-fourth strength liquid medium with 2% sucrose on a rotary cultivation apparatus (2 cycles per minute)

at 26±0.5 °C under 505 lux continuous fluorescent illumination. The following modifications of the standard Gamborg B5 medium (Gamborg *et al.*, 1968; in mg.l^{-1}: KNO_3, 2500; $(NH_4)_2SO_4$, 134.0; NaH_2PO_4, 130.5; $CaCl_2.6H_2O$, 223.5; $MgSO_4.7H_2O$, 250.0; $FeSO_4.7H_2O$, 27.8; Na_2EDTA, 37.3; H_3BO_3, 3.0; $MnSO_4$, 10.0; $ZnSO_4.7H_2O$, 2.0; $CuSO_4.5H_2O$, 0.025; $CoCl_2.6H_2O$, 0.025; $Na_2MoO_4.2H_2O$, 0.25; KI, 0.75; inositol, 100; thiamine, 10.0; nicotinic acid, 1.0; pyridoxine, 1.0) were tested.

A, full-strength standard B5 + 2500 mg.l^{-1} KNO_3; B, 50% B5 + 200 mg.l^{-1} KNO_3; C, 50% B5 + 500 mg.l^{-1} KNO_3; D, 50% B5 + 1000 mg.l^{-1} KNO_3; E, 50% B5 + 0 mg.l^{-1} KNO_3; F, 50% B5 + 500 mg.l^{-1} KNO_3 + 100 mg.l^{-1} peptone; G, 50% B5 + 500 mg.l^{-1} KNO_3 + 300 mg.l^{-1} peptone; H, 50% B5 + 500 mg.l^{-1} KNO_3 + 1000 mg.l^{-1} peptone; I, 50% B5 + 500 mg.l^{-1} KNO_3 + 20 mg.l^{-1} glycine; J, 10% B5 + 20 mg.l^{-1} KNO_3. All media contained 2% sucrose and their pH was adjusted to 5.5 (NaOH, HCl) before autoclaving.

Four non-branched 2.0±0.2 cm long apical segments from shoots of E Polish (mean plant dry weight 5.1 mg) or 1.5±0.2 cm long segments of Japanese *Aldrovanda* plants (mean plant dry weight 0.81 mg) were inserted aseptically into each of 3 cm diameter, 100 ml test tubes into 40 ml of the media. Each variant of the media consisted of three parallel test tubes for each *Aldrovanda* strain. The experimental plants were grown under the same conditions as above. Shoot apices of the Polish plants were counted without opening the test tubes after 14 days of growth. The growth experiment was terminated after 22 days for Polish plants and 28 days for Japanese plants. The following characteristics were estimated: total shoot apices per tube, shoot length, number of adult leaf whorls on main shoots, maximum trap size, dry weight per tube, and pH of used media. Browned senescent leaf whorls were discarded. The data were expressed as mean (±1 SE) of three parallel test tubes and analyzed using ANOVA (Tukey HSD test). H^+ concentrations were used for statistical treatment instead of pH values.

During the whole growth experiment, plant dry weight increased on average by 1.9-5.9 fold in Polish plants (Table 1) and by 2.2-5.2 fold in Japanese ones (Table 2). In Polish plants growing both in the full-strength B5 basal medium and in one-tenth-strength B5 medium with only 20 mg.l^{-1} KNO_3, branching of shoots ceased as early as 14 days of growth, while all the other media were able to further support statistically significantly (at $P<0.05$) branching of shoots. In Polish plants growing in half-strength B5 medium with different KNO_3 concentrations, the medium with 1000 mg.l^{-1} KNO_3 was the best in all characteristics though the statistical significance was rather weak (Table 1B-E).

The addition of 100-1000 mg.l^{-1} peptone to half-strength B5 medium with 500 mg.l^{-1} KNO_3 for Polish plants led to a step-wise increase of all growth characteristics but usually only the effect of 300 and 1000 mg.l^{-1} peptone was statistically significantly different from the absence of peptone (Table 1C, F-H). The variant with 1000 mg.l^{-1} peptone was clearly the best of all tested media for the growth of Polish plants. On the other hand, the addition of 20 mg.l^{-1} of glycine was quite without any effect on plant growth (Table 1C, I). Similarly, addition of certain amino acids (pro, hypro, val, met, leu, ile, lys, ala) to an agar-based medium had no effect or a negative effect on the growth of *Drosera rotundifolia in vitro* (Simola, 1978). In Polish plants except for the full- and one-tenth-strength B5 medium, mean pH values in all other used media were between 2.85 and 3.06 (Table 1). Higher KNO_3 concentrations corresponded to slightly higher pH values.

Growth characteristics of Japanese plants in various media were slightly different from those of Polish plants (Table 2). The worst variant was clearly the standard full-strength B5 medium. No clear relationship was found between growth characteristics and KNO_3 concentration in a half-strength B5 (Table 2B-E). However, within these variants, zero KNO_3 concentration was the best. Similar growth was attained in one-tenth-strength B5. The addition of 100-1000 mg.l^{-1} peptone to a half-strength B5 medium with 500 mg.l^{-1} KNO_3 led to a two- to threefold increase in the number of shoot apices but, due to large variance in the data, these differences were usually not statistically significant (Table 2C, F-H). Out of all variants, that with 300 mg.l^{-1} peptone was clearly the best (number of shoot apices and leaf whorls, shoot length, and dry weight) for the growth of Japanese plants. As in Polish plants, the addition of 20 mg.l^{-1} of glycine was quite without any effect on plant growth (Table 2C, I). pH values in used media ranged from 3.14 to 3.76, except for the medium with 1000 mg.l^{-1} peptone (pH 4.47).

Generally, Japanese *Aldrovanda* plants in a rotary *in vitro* culture are much smaller and thinner than E Polish plants (Kondo *et al.*, 1997), and the same relation was confirmed by the present results (cf. Tables 1 and 2). According to these authors, this difference is genetically

based. However, in a stagnant *in vitro* culture in a half-strength B5 medium, their size and features are the same (Pásek and Adamec, unpublished).

The optimal KNO_3 concentration in half-strength B5 medium for growth of both Polish and Japanese *Aldrovanda* plants may be 500-1000 $mg.l^{-1}$. Such high concentrations are not necessary for total mineral N uptake by the plants but they partly counterbalance medium acidification due to dominant NH_4^+ uptake. However, KNO_3 concentration could also have a direct effect on organogenesis as recently reported by Idei and Kondo (1998) in carnivorous *Utricularia praelonga* grown in a shoot primordium culture. The preference of Japanese *Aldrovanda* plants for one-tenth-strength diluted B5 medium or for low KNO_3 concentration (Table 2) may not represent specific properties of this strain but it may reflect the fact that the media were not exhausted at the end of the growth experiment, as opposed to Polish plants. During the growth experiment, small Japanese plants increased their dry biomass by only 4-15 mg per tube, while robust Polish plants did so by 18-100, on average ca. 50 mg per tube. In 40 ml of KNO_3-free half-strength B5 medium in one tube, there was only 0.57 mg of NH_4^+-N available. The mean 50-mg growth increment could contain ca. 0.60 mg N and 1.1 mg K in the biomass (ca. 1.2 % N and 2.2 % K in dry biomass; Adamec and Pásek, 2000). It means that the growth of Polish plants in KNO_3-free a half-strength B5 or a tenth-strength B5 medium + 20 $mg.l^{-1}$ KNO_3 (Table 1E, J) was evidently limited by a shortage of N, but much more so by low levels of K^+ in the tubes. Thus, the advantage of intermediate KNO_3 concentrations (500-1000 $mg.l^{-1}$) in half-strength B5 medium was based on much greater N and K^+ supplies supporting both greater growth increments and reaching higher pH values. However, pH values below 3.0 were not toxic for the growth of *Aldrovanda in vitro.* In both strains of *Aldrovanda* tested, the full-strength B5 medium with 2500 $mg.l^{-1}$ KNO_3 appeared to be excessively concentrated for optimal growth (Tables 1 and 2; Adamec and Pásek, 2000).

An addition of peptone to the standard medium containing half-strength B5 with 500 $mg.l^{-1}$ KNO_3 has shown to be positive for the growth of the both *Aldrovanda* strains. The peptone concentrations of 300-1000 $mg.l^{-1}$ were the best in the both strains (Tables 1 and 2). Since peptone as an extract of beef contains ca. 16 % of organic N the relatively large growth enhancement due to the addition of peptone proves the great capacity of *Aldrovanda* for the uptake of nitrogenous organic substances instead of mineral forms of N (cf. Adamec, 1997b).

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Table 1. The *in vitro* growth of *Aldrovanda vesiculosa* from E Poland in modified Gamborg B5 media after 22 days

Var.	Total shoot apices per tube after		Shoot length	Leaf whorls of main shoot	Dry weight per tube	Max. trap size	Final pH of medium (range)
	14 d	22 d	(cm)		(mg)	(mm)	
A	6.3±0.3^{ab}	6.3±0.3^{a}	6.4±0.4^{a}	26.9±1.9^{ab}	65.3±3.8^{ac}	3.0±0.0^{b}	3.42(3.35-3.47)^{a}
B	5.0±0.0^{a}	8.00±0.6^{ac}	6.4±0.4^{a}	25.5±3.0^{a}	72.0±3.0^{ac}	3.0±0.0^{b}	2.91(2.85-2.99)^{bd}
C	4.7±0.7^{a}	7.0±0.6^{ac}	7.0±0.2^{a}	27.3±0.8^{ab}	64.7±9.7^{ac}	3.0±0.0^{b}	2.96(2.90-3.02)^{bd}
D	5.0±0.6^{a}	8.3±0.3^{ac}	8.5±0.1^{bc} d	33.4±1.7^{b}	82.3±7.6^{ae}	3.2±0.2^{bc}	3.06(3.02-3.11)^{b}
E	5.7±0.3^{ab}	7.0±0.0^{ac}	7.4±0.2^{ac}	29.9±0.6^{ab}	66.3±5.0^{ac}	3.0±0.0^{b}	2.85(2.80-2.88)^{cd}
F	6.0±0.6^{ab}	10.3±0.9^{bc} d	9.0±0.1^{d}	31.9±0.7^{ab}	88.0±7.9^{ad} f	3.0±0.0^{b}	2.93(2.90-2.96)^{bc}
G	5.3±0.3^{a}	12.7±0.9^{b}	9.6±0.1^{de}	33.8±0.6^{b}	91.3±3.7^{ad}	3.3±0.2^{bc}	2.93(2.89-2.96)^{bc}
H	8.3±0.7^{b}	14.0±1.2^{b}	10.6±0.3 e	35.2±1.0^{b}	120.0±7.5 bd	3.5±0.0^{c}	3.06(2.99-3.14)^{b}
I	4.3±0.3^{a}	6.7±0.3^{ad}	6.4±0.1^{a}	28.5±1.4^{ab}	53.3±2.4^{ce} f	3.0±0.0^{b}	3.05(3.04-3.06)^{b}
J	5.0±0.6^{a}	5.0±0.6^{a}	5.9±0.2^{a}	24.5±0.4^{a}	37.7±2.0^{c}	2.5±0.0^{a}	3.47(3.46-3.48)^{a}

A, full-strength B5 + 2500 mg.l^{-1} KNO_3; B, 50% B5 + 200 mg.l^{-1} KNO_3; C, 50% B5 + 500 mg.l^{-1} KNO_3; D, 50% B5 + 1000 mg.l^{-1} KNO_3; E, 50% B5 + 0 mg.l^{-1} KNO_3; F, 50% B5 + 500 mg.l^{-1} KNO_3 + 100 mg.l^{-1} peptone; G, 50% B5 + 500 mg.l^{-1} KNO_3 + 300 mg.l^{-1} peptone; H, 50% B5 + 500 mg.l^{-1} KNO_3 + 1000 mg.l^{-1} peptone; I, 50% B5 + 500 mg.l^{-1} KNO_3 + 20 mg.l^{-1} glycine; J, 10% GB5 + 20 mg.l^{-1} KNO_3. Mean values ±1 SE interval are stated in all cases. The data are means of three parallel test tubes. Within each column, the variants labelled by the same letters are not statistically significantly different at $p<0.01$.

Table 2. The *in vitro* growth of *Aldrovanda vesiculosa* from Japan in modified Gamborg B5 media after 28 days

Var.	Total shoot apices per tube	Shoot length (cm)	Leaf whorls of main shoot	Dry weight per tube (mg)	Max. trap size (mm)	Final pH of medium (range)
A	7.7±3.7^{a}	2.0±0.04^{a}	2.3±0.2^{a}	7.7±1.7^{a}	2.3±0.1^{a}	3.76(3.64-3.96)^{ac}
B	6.7±0.3^{a}	2.6±0.1^{bc}	4.9±0.2^{ab}	11.7±1.9^{ab}	2.1±0.1^{ab}	3.36(3.28-3.35)^{ag}
C	15.3±4.2^{abf}	2.6±0.1^{bc}	5.1±0.1^{ab}	12.7±0.5^{ab}	1.9±0.1^{ab}	3.34(3.29-3.38)^{ag}
D	11.3±3.5^{ab}	2.5±0.03^{b}	4.6±0.2^{ab}	10.3±1.5^{ab}	2.0±0.1^{ab}	3.43(3.36-3.53)^{a}
E	18.7±5.0^{abc}	3.0±0.1^{bc}	6.1±1.0^{ab}	12.0±0.6^{ab}	1.7±0.1^{ab}	3.14(3.06-3.26)^{bg}
F	29.7±5.2^{bd}	2.7±0.1^{bc}	6.4±1.0^{ab}	13.9±2.9^{ab}	2.0±0.1^{ab}	3.49(3.34-3.62)^{ade}
G	42.3±2.9^{d}	3.0±0.1^{c}	8.4±0.8^{b}	18.3±0.3^{b}	1.9±0.2^{ab}	3.60(3.53-3.67)^{adf}
H	32.7±0.9^{cdef}	2.8±0.1^{bc}	6.2±1.3^{ab}	11.7±1.2^{ab}	2.0±0.1^{ab}	4.47(4.33-4.59)^{cefh}
I	14.0±1.7^{abe}	2.7±0.1^{bc}	4.3±0.4^{ab}	12.0±0.2^{ab}	1.5±0.1^{b}	3.56(3.46-3.72)^{adh}
J	17.3±1.3^{abe}	2.7±0.1^{bc}	6.8±1.0^{ab}	17.0±0.6^{b}	1.5±0.3^{ab}	3.38(3.37-3.39)^{ad}

For the description of the media and further details see Table 1.

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Effects of introduction of *Aldrovanda vesiculosa* L. in Poland

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Aldrovanda vesiculosa L. (family *Droseraceae*) is cosmopolitan species. It is come within a legal protection in Poland and placed in "Red List".

Historical and actual sites according to the newest data are shown in the table.

State in year 2000	Number of sites			
	out-of-date and (of exacting verification)	existing nowadays		total
		natural	supplementary and restored	
World	136 + (90)	47	21	293
Europe (without Poland)	50 + (50)	22	7	128
Poland	82 + (2)	7	13 + (7 ?)	104

In Poland were most of all known sites (together 92 sites, 50 actual sites in year 1958). In 1990 existed in nine sites. Only four or five sites from them promised survival in near time (in the last 12 years disappeared from two sites).

Works on active protections of *A. vesiculosa* were concentrated

1. on obtainment in cultivation the greatest number of plants to testing of selected sites and next to introduction them together with elaboration of more effective technics of reproducing of *A. vesiculosa* (cultures "*in vitro*"),
2. on choice of natural sites and show suitable habitats and test them using controlled cultivations of *A. vesiculosa*,
3. on plants introduction to the best from checked sites after acceptance of proper administrative organs.

In years 1992-1997 inspected near 250 sites in Poland. From different reasons over 190 sites were verified negatively. 55 inspected sites had physical, chemical and biotic (suitable composition of accompanying plants) features of proper habitats of *A. vesiculosa.* Through two years were prepared cultivations (baskets) of aldrovanda. Over half of sites did not realize put in them of hope. Every year *A. vesiculosa* died in 21 sites but in three sites survived but growth was negative. On 11 sites baskets were systematically destroyed. It has been stated, that in 20 sites every year growth of number of aldrovanda increased ie. from 25% to 1400 % a year. So, to those sites tens or several hundred of shoots were introduced. All sites were checked in 2000 year. In 8 sites (ie. 14 % tested sites, but 3 % checked sites) populations of *A. vesiculosa* number was from thousand to several tens thousand of shoots and they are in progressive phase. In the remaining 12 sites the number was from tens to several hundred of individuals. Unfortunately in seven sites the growth of *A. vesiculosa* shoots was poor and can to disappear.

Table placed above shows proportion of restored sites to natural ones (2:1).

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See the address http://bestcarnivorousplants.com/aldrovanda for references on *Aldrovanda vesiculosa* (history, ecology, biology, physiology, protection and cultivation).

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Extraction and purification of high quality DNA for PCR direct sequence in endangered *Aldrovanda vesiculosa* L.

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Abstract. DNA extraction and purification of endangered *Aldrovanda vesiculosa* were performed to produce PCR product and to determine nucleotide sequence. To develop DNA extraction protocol, fresh and dried plant tissues were provided from *in vivo*- and *in vitro*-cultured plant materials. A CTAB- extraction method was employed to isolate total genomic DNA from the species. After establishing the protocol to isolate high quality DNA, the region of nuclear 5.8S and internal transcribed spacer (ITS) ribosomal DNA (rDNA) were amplified by polymerase chain reaction (PCR). The PCR products were purified by gel-cutting method, and were treated by PCR direct sequence method. Using these methods, the 5.8S and ITS rDNA sequences of six populations in *A. vesiculosa* were determined. A comparative study with these sequences was carried out to infer genetic diversity in *A. vesiculosa*.

Introduction

A monotypic genus *Aldrovanda* L., which belongs to the Droseraceae, contains a single aquatic carnivorous plant species producing insectivorous leaf with snap trap mechanism similar to genus *Dionaea*. This aquatic species, *Aldrovanda vesiculosa* L., is widely but sparsely distributed throughout the Old World and Australia. *Aldrovanda vesiculosa* is sensitive to acid rain, eutrophication, floods, and other human disturbances (Kondo *et al.*, 1997). Due to these environmental changes in last several decade, some local races of the species have vanished from the earth. It was extinct from the nature in at least six countries (Kondo *et al.*, 1997). Thus, *in vitro* culture is one of the most important methods to maintain the potentially-valuable gene pool of such plants (Bobak *et al.*, 1995, Kondo *et al.* 1997, Samaj *et al.*, 1999). Moreover, the stocks of high quality DNA are a prerequisite for molecular biological studies in endangered plants (Bekesiova *et al.*, 1999).

In this study, an attempt of DNA extraction and purification from *in vivo*- and *in vitro*-cultured *Aldrovanda vesiculosa* were performed to produce PCR product and to determine nucleotide sequence. After establishing the protocol to product DNA sequencing samples, on nuclear 5.8S and internal transcribed spacer ribosomal DNA sequences of six populations of *A. vesiculosa* were determined. With these sequences, a comparative study was carried out to infer genetic diversity in *A. vesiculosa*.

Materials and Methods

Plant materials The *in vivo* living materials of six populations from Eastern and

Northern Australia, Italy, Japan, Poland and Ukraine of *Aldrovanda vesiculosa* were supplied form Laboratory of Plant Chromosome and Gene Stock, Graduate School of Science, Hiroshima University. Additionally, two *in vitro*-culture strains from Japan and Poland were used.

Extraction of genomic DNA Total DNA extraction mainly followed Hasebe *et al.* (1994). The plant tissues were ground into powder with liquid nitrogen and homogenized in the buffer containing 0.1M Tris-HCl (pH 8.0), 20 mM EDTA (pH 8.0), 1.4M NaCl, 2% cetyltrimethylammonium bromide and 0.5% mercaptoethanol. The homogenate was extracted three times with an equal volume of chloroform-isoamyl alcohol (24 : 1) for 15 minutes each and the DNA was precipitated with an equal volume of isopropyl alcohol at room temperature.

Polymerase chain reaction (PCR), DNA purification, and DNA sequences The amplification protocol was mainly followed Hasebe *et al.* (1994). For PCR amplification of nuclear 5.8S and internal transcribed spacer (ITS) ribosomal DNA (rDNA), reaction mixtures were made up from equal volumes of DNA solution and a master mix containing all the other necessary components. Final concentrations were: 10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 200 μ M of each of the four deoxyribonucleotide triphosphates, 0.5 μM of each of two different primers, 25 U/ml *Taq* polymerase (Takara Co. LTD.), and less than 1 μ g template DNA. The amplified products were electrophoresed on 0.7 % agarose gels and product bands were cut out from the gels. Then, they were purified with GeneClean III (BIO 101 Inc., La Jolla, CA). The purified double-stranded DNA was sequenced in both directions using a BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA) and ABI PRISM 377 DNA sequencer (Applied Biosystems). The both ITS regions including the 5.8S rDNA nucleotide sequences were aligned using Clustal W version 1.8 (Thompson *et al.*, 1994), and then revised manually.

Results and Discussion

Extraction of genomic DNA The result of total DNA extraction was shown in Fig. 1. Ethidium bromide-stained agarose gel of total genomic DNA showed that large amounts of total genomic DNA were obtained from *in vitro*-cultured *Aldrovanda vesiculosa* plant materials. On the other hand, *in vivo*-cultured *A. vesiculosa* plant provided small amount genomic DNA. *Drosera spathulata*, belongs to the same family of Droseraceae, was used as control comparison. Even though the *in vitro*-cultured plant material were used, *Drosera spathulata* provided small amount genomic DNA in this extraction methods. Bekesiova *et al.* (1999) mentioned that difficulty to isolate DNA from *Drosera* was due to the presence of large numbers of upper-leaf surface glands contained a shining, highly viscous mucilage. Our comparative results of total genome DNA between *Aldrovanda vesiculosa* and *D. spathulata in vitro* plants supported inference of Bekesiova *et al.* (1999).

PCR amplification and DNA purification Nuclear 5.8S and internal transcribed spacer (ITS) ribosomal DNA (rDNA) sequences were amplified by PCR. The result of PCR amplification was shown in Fig. 2. Single PCR product band was appeared in *in vitro*-cultured *Aldrovanda vesiculosa* plant materials. Thus, The obtained DNA from *in vitro*-cultured plant of *A. vesiculosa* was of sufficient quality for PCR. In contrast, total genome DNA of *Drosera spathulata* made many faint PCR product bands, but not any clear targeting band. Two PCR products were amplified from Australian population of *Aldrovanda vesiculosa*. According of the public sequence databases, the PCR-product band with higher molecular weight (> 1.0 kb) in Australian population might have sequence of other

microorganisms such as water algae. In general, the genomic DNA from most water algae species could product more than 1.0 kb size of the band with same PCR primer set.

The result of PCR product purification was shown in Fig. 3. By the gel cutting method, only target DNA band could be isolated from PCR products. The gel cutting step in purified protocol was prerequisite to remove a contamination of other organism's DNA from *in vivo*-cultured plant DNA in *Aldrovanda vesiculosa*. The contaminated PCR production is the most unavoidable problem for *in vivo* aquatic plant. This problem imply that establishment of *in vitro*-grown system in aquatic plant materials was extremely essential to supply valuable gene pools or plant molecular biological tools such as cDNA and genomic library construction.

It is extinct from the nature in Bangladesh, Denmark, France, Germany, Italy, Japan, and other places. Meanwhile, it is endangered in Poland, Switzerland, and vulnerable in Bulgaria, China, Croatia, the Czech Republic, Hungary, and Russia (Kondo *et al.*, 1997). In Japanese extinct population, only cultivated plants were exist and strictly protected as a national natural monument. Since many of local races of *Aldrovanda vesiculosa* has become endangered or were extinct, importance of valuable DNA stock and gene pool of *Aldrovanda vesiculosa* would increase in further molecular analysis and conservation biology.

DNA sequence determination The sequences result of *Aldrovanda vesiculosa* was shown in Fig. 4. Among six populations in *A. vesiculosa*, completely homologue sequence result was supplied from Clustal W analysis. In contrast to the sequence arraignment result in *Aldrovanda vesiculosa*, recent study showed that *Drosera spathulata* had DNA polymorphism of nuclear 5.8S ITS rDNA sequences even in Japanese tetraploid populations (Hoshi *et al.* under preparation).

A morphological interspecific polymorphisms have been found in some species in the Droseraceae. *Aldrovanda vesiculosa* is one of the interest species for interpecific morphological polymorphism (Adamec 1999) as well as the *Drosera spathulata* complex (Hoshi *et al.*, 1994). *Aldrovanda vesiculosa* has discontinuous distribution pattern in Old world and Australia, speculating the difficulty of genetic exchange. Moreover, ancestor genus of *Aldrovanda* is the oldest in the carnivorous plant and might occurred during the end of the Cretaceous period (Senonian epoch, from 85 to 75 million years ago) (Knobloch and Mai, 1984; Degreef, 1997). However, the DNA sequence result indicated that ITS sequence of extant *Aldrovanda vesiculosa* was very conservative and might have slower evolution than that of *Drosera spathulata*. The differences of genetic diversities between two species led us to offer three following hypothesis: (1) *Aldrovanda vesiculosa* might be a new formed species, (2) evolution rate of *Aldrovanda vesiculosa* might be slower than that of other species in the Droseraceae, or (3) genetic drift have occurred in *Aldrovanda vesiculosa*. In the European population of *Aldrovanda vesiculosa,* the origin of this population was suggested that seeds or turions of the species were transported by migratory birds (Adamec, 1999). Since the spread to new sites might be due to waterfowl as migration-enhanced factor, the hypothesis of genetic drift would be supported.

Acknowledgement

The authors gratefully acknowledge the technical support from Dr. Ryosuke Sano for molecular experiments.

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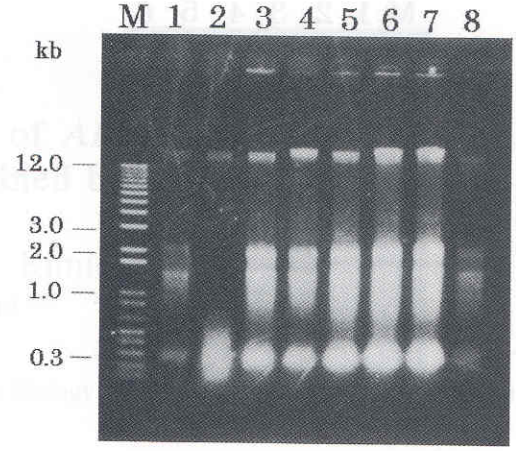

Fig. 1 Ethidium bromide-stained 1 % agarose gel of total genomic DNA from *Drosera spathulata* (1 and 2) and *Aldrovanda vesiculosa* (3 - 8). *Drosera spathulata*, belongs to the same family of the Droseraceae, was used as control comparison. All samples were obtained from *in vitro* culture, except for Australian population (8) of *Aldrovanda vesiculosa*. Fresh (1, 4 and 8) and dried (2, 3 and 5-7) plant tissues were provided from Australia (8), Japan (3 -5) and Poland (6 and 7) populations of *Aldrovanda vesiculosa*, and New Zealand (1 and 2) population of *Drosera spathulata*. Size marker (M) was given in kb.

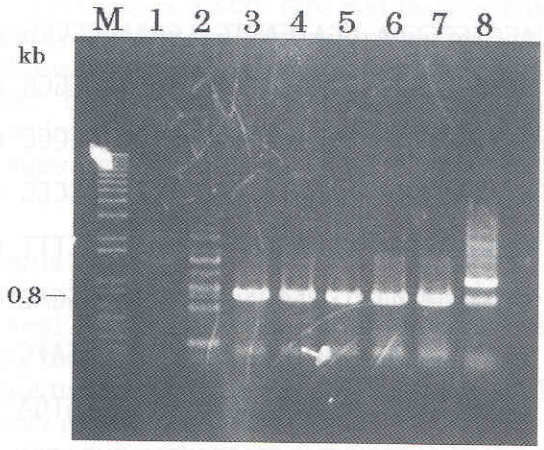

Fig. 2 Ethidium bromide-stained 1 % agarose gel of a PCR with total genomic DNA from *Drosera spathulata* (1 and 2) and *Aldrovanda vesiculosa* (3 - 8). Size marker (M) was given in kb. The templates were total genomic DNA of Australia (8), Japan (3 - 5) and Poland (6 and 7) populations of *Aldrovanda vesiculosa*, and New Zealand (1 and 2) population of *Drosera spathulata*. Two PCR products were amplified in Australian population of *Aldrovanda vesiculosa*. According of the public sequence databases, the PCR-product band with higher molecular weight (> 1.0 kb) in Australian population might have sequence of other microorganisms such as water algae. Total genome DNA of *Drosera spathulata* made many faint PCR product bands, but not any clear targeting band.

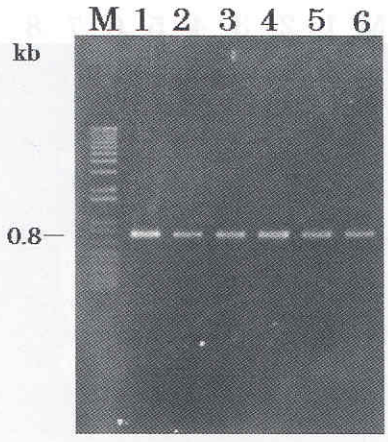

Fig. 3 Ethidium bromide-stained 1 % agarose gel of purified DNA of the PCR products. The purified PCR products were obtained from total genomic DNA of Australia (6), Japan (1 - 3) and Poland (4 and 5) populations of *Aldrovanda vesiculosa*, and New Zealand (1 and 2) population of *Drosera spathulata*. Size marker (M) was given in kb. By the gel cutting method, only target DNA band could be isolated from PCR products.

```
  1  CCGTAGGTGA ACCTGCGGAA GGATCATTGA CGAAACCCCG ACCCTTTGGC   50
 51  ACCCCCGTCG AGAACGAGTA AAGGAATCCC AGCTGGCGGG ATGGTGTGCT  100
101  TGTCCCAGGC ACGCCTTCTC TCCGGTTGTC GTGCTGGCCC ATCTTCCGCG  150
151  GCACCGTCCG GTAGAGCGGA AAGGTGTGCG CCTGGCGCGG CGACTTAACA  200
201  ACAATCAGGC GCGGGATGCG CCAAGGACAA TGGATGCTTT GACATGCCTT  250
251  TGCCCCTTCT GTCTGGTTCT TGGACCAGGG GATAGGGGAC GGCAATGCGC  300
301  TGTACGCGCG AGATTGACAC GACTCTCGGC AACGGATATC TCGGCTCTCG  350
351  CATCGATGAA GGACGTAGCG AAATGCGATA CTTGGTGTGA ATTGCAGAAT  400
401  CCCGTGAACC ATCGAGTCTT TGAACGCAAG TTGCACCCGA GGCCACCAAG  450
451  GCTAAGGGCA CGTCTGCCTG GGTGTCACCT AGACCCTCAC CAAACCCGGG  500
501  TTCACTTCAG CGGAAGTGGC GGGGGGTGGT GGACGATGGC CTCCCGTGCT  550
551  CCTCGTGCGG TTGGCCGGAA TCTGGAGCTG GAGCCGTTGA GCGCAGCGGC  600
601  GTATGGTGGT TGGGCGAGGA GAAAGTCCTC GATTCGTCCC CCCCCCGTTG  650
651  AGACTCGCCG CTGCGACAGC GCGTGCCCGA TTCCCGCGCT ATCCCTATCG  700
701  AGCC
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Fig. 4 Nucleotide sequence of nuclear 5.8S and internal transcribed spacer ribosomal DNA of *Aldrovanda vesiculosa*. There was no differences among the sequences of six populations in *Aldrovanda vesiculosa*.

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C-4

Fine structure of *Aldrovanda vesiculosa* during the life cycle examined by light and electron microscopy

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Abstract. Unique fine structural features of the *Aldrovanda vesiculosa* shoot apex, flower, seed formation and seed germination were revealed by light and electron microscopic observation.

Introduction

Since *Aldrovanda vesiculosa* is a very rare and inaccessible plant, details of its fine structure during the life cycle were not known. In this research, we aimed to reveal its fine structure. The *Aldrovanda vesiculosa* used in this study was maintained ex-vitro after in vitro cultivation and multiplication. Fine structure of shoot apex, flower, seed formation, seed germination, and carnivorous leaves including digestive glands, sensory hairs and absorptive hairs developed on them were observed using light microscopy, SEM and TEM.

Materials and methods

Aldrovanda vesiculosa was cultured in vitro and maintained ex-vitro. Fine structure of its shoot apex, flower, seed formation, seed germination and the carnivorous leaf was studied by the following methods.

- the direct SEM viewing method (Saito *et al.*, 1995; Arai *et al.*, 1996)
- light microscopic observation of seed sections
- TEM observation of ultra thin sections of carnivorous leaves prepared by conventional chemical fixation and rapid freezing method

Results and Discussion

By SEM observation, the shoot apex of *Aldrovanda vesiculosa* was found to be exposed, not covered by leaf primordia (Fig.1; SA: shoot apex, CL: developing carnivorous leaf). Observation of flower structures by SEM showed that, stamens (S) and pistil (P) were closely situated (Fig.2) and developing seeds were seen in the ovary (Fig.3). Fig.4 shows a section of a seed about to germinate observed by light microscopy (C: cotyledon, E; endosperm, R: radicle, arrow: shoot apex). TEM observation of the ultra thin sections revealed that endosperm contains amyloplasts and protein bodies for storage, while the embryo contains lipid bodies and amyloplasts. On young carnivorous leaves, developing digestive glands (D), sensory hairs (S), and absorptive hairs (A) were observed (Fig. 5). Fig. 6 shows an SEM image of developing digestive glands. TEM observation of each gland and hair cell revealed development of ER, vacuoles and Labyrinthin walls (Juniper *et al.*, 1989) in digestive glands (Fig.7; N: nucleus, V: vacuole, arrow: labyrinthin wall), abundant plasmodesmata and ER in

sensory hairs (Fig.8; N: nucleus, arrow: plasmodesmata), and proliferation of Golgi bodies in absorptive hairs (Fig.9; GB: Golgi body, prepared by rapid freezing).

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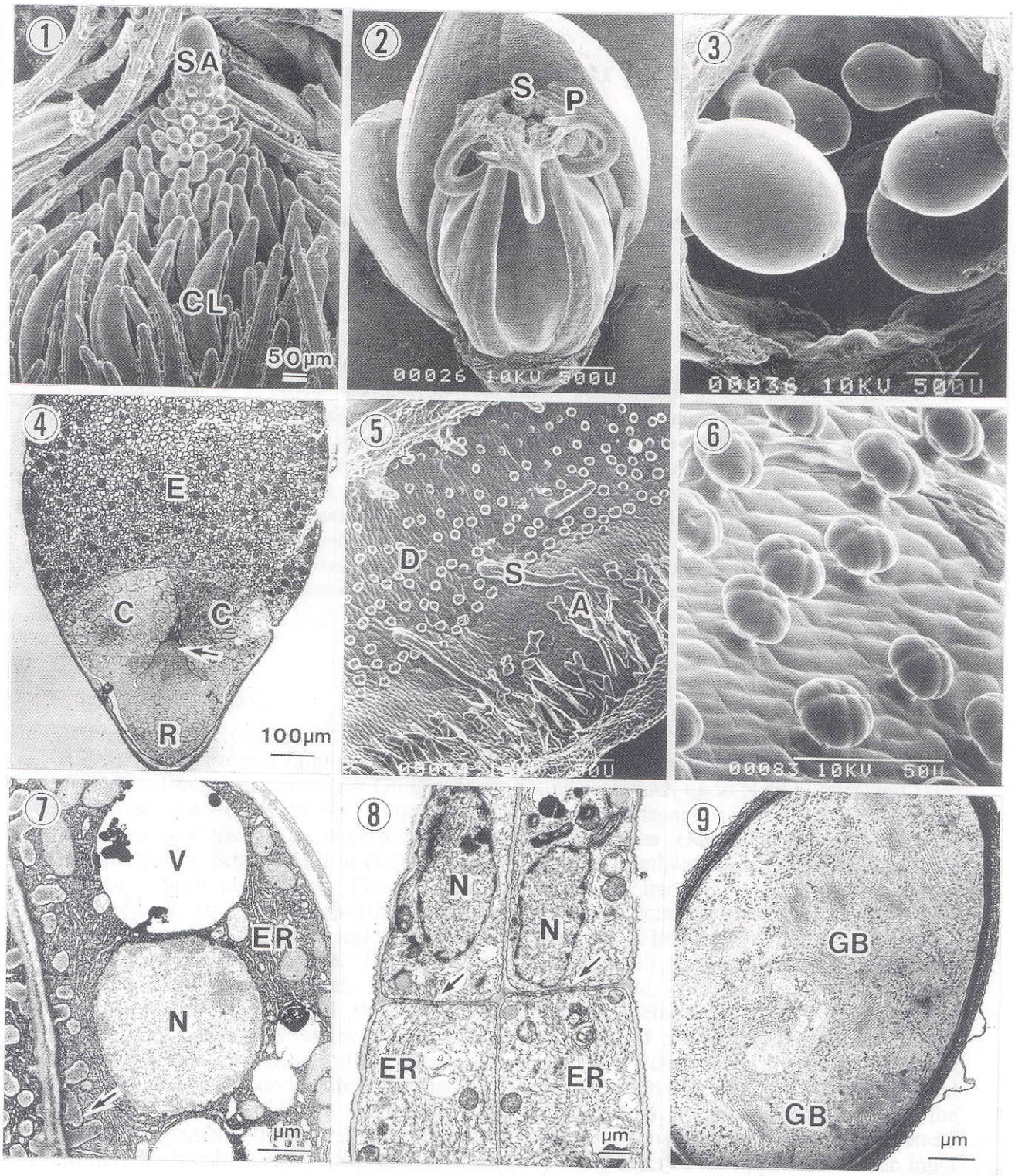
①
SA
CL
50μm
②
S
P
00026 10KV 500U
③
00036 10KV 500U
④
E
C
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100μm
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S
A
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D-1

Ecology and diversity of pitcher plants in Sarawak

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ABSTRACT. The study to determine the diversity and the distribution of *Nepenthes* in Sarawak base on the examination of herbarium specimens as been carried out. Endemic species recognized includes *N. bicalcarata, N. curtisii, N. faizaliana, N. fusca, N. hirsuta, N. lowii, N. muluensis, N. northiana, N. pilosa* and *N. veitchii*. Pitcher plants recorded outside Borneo identified include *N. albomarginata, N. ampullaria, N. gracilis, N. mirabilis, N. rafflesiana, N. reinwardtiana* and *N. tentaculata*. Species with rare occurrence or confined to a particular type of forest are *N. northiana* (Bau and limestone endemic), *N. muluensis* (Mount Mulu and Mount Murud), *N. faizaliana* (Bukit Panjang in Mulu), *N. fusca* (Mt. Apo in Kelabit Highland). Seven species occurring outside Borneo are generally widely distributed in Sarawak. The habitat depending on species can be found growing on roadside clearing, heath forest, secondary scrub, peat swamp forest, forest underlying ultrabasic and limestone outcrop, gap of dipterocarp primary forest, mossy forest.

INTRODUCTION

Nepenthes belonging to family Nepenthaceae contained 81 species in the world (Adam, 1995). This tropical family has the center of distribution in Borneo, Malay Peninsular, Sumatra, Philippines and New Guinea. It can found eastwards up to New Guinea and Isles of Pines, westwards to Sri Lanka, Seychelles and Madagascar, absent in the African continent, southwards it can be found in the York Peninsular, Queensland in Australia, and northwards to Khasia Hill in Assam, Indo-China and Southern China. Pitcher plants are climbers and scramblers. They are terrestrial plants but some species may grow epiphytically on trees. One of the main characters of the genus is the presence of pitchers variously shaped, colored, and sized on their tendril tips. Their flowers are arranged along the axis called inflorescence. The plants are dioecious, their male and female inflorescence are borne on different plants. The flowers secreted nectar from the glands on the upper surface of the flower sepal; the nectar emitted a weak foetid smell as to attract potential pollination agents such as the ants and flies. The ability of pitcher plants growing in low nutrient soil is attributed to their carnivorous habit. It is achieved by the modification of leaves into pitchers on the tip of the tendrils. The pitchers have the ability to attract potential prey by variously colored, shaped and sized of the pitchers, secretion of nectar from the lid nectar glands and marginal glands situated in between peristome teeth. The effectiveness of the trap is enhanced by the presence of slippery waxy layer of the upper half on the inner pitcher cavity in most but not in all species.

Materials and Methods

Information on the ecology, altitudinal distribution, localities and morphological similarity and differences between species were obtained from the herbarium specimens examined from various herbaria: K, L, BO, UKMB, UKMS, SAN, SAR, SING, FRI, BRIS, and my personal collection from Sarawak. The information for endemic species and species occurring outside Borneo was gathered from field observations, and aided by literatures (Macfarlane, 1908; Danser, 1928; Holttum, 1940; Kurata, 1976; Turnbull and Middleton, 1981; Hotta and Tamin, 1986; Som, 1988; Adam *et al.*, 1992)

Results and Discussion

Endemic species

Nepenthes bicalcarata This species is very closely related to *Nepenthes ampullaria* but differ from this species and other species of the family by the presence of two incurved thorns on the lid base of upper and lower pitchers. It is recorded in Sarawak from Betong Forest Reserve, Baram, Loba, Kavang South Forest Reserve, Penyilam Bintulu, Lawas River, Merapok and Marudi. It occurs from about 10 m to 30 m altitude in disturbed primary forest, in after-logged forest growing together with *Nepenthes ampullaria*.

Nepenthes curtisii This species has been collected from Gunung Silantek in Sri Aman, Carapa Pilla Mujong Watershed in Balleh, Gunung Api, Bukit Banggai at Sungai Lemanak, Bukit Sekanjang in Lanjak Entimau, Mount Penrissen, Gunung Pueh in Lundu, Mount Mulu, Mount Berumput, Mount Poi and Mount Murud. This highland species occurs from 900 m up to 1650 m altitude in mossy forest, exposed mountain ridges, montane scrub vegetation. This species is morphology related to *Nepenthes fusca* by the presence of glandular crest at the base of the pitcher lid below but differs by the presence of extended apical appendages of lower lid surface.

Nepenthes faizaliana This species is found from Bukit Panjang in Mulu. It is morphologically similar with *Nepenthes fusca* by its infundibulate upper pitcher, presence of glandular crest on the lower lid surface, semi-amplexicaul leaf base but differs in having two-flowered pedicels and partly glandular upper pitcher.

Nepenthes fusca This species has very rare occurrence, being recorded on Summit of Mount Apo Dari in Kelabit Highland. It is similar with *Nepenthes curtisii* by the presence of glandular crest on the lower lid surface but differs by the absence of extended apical glandular appendages on lid surface below.

Nepenthes hirsuta This species has been collected from Bungoh Range, Teluk Pasir in Santubong, Sungai Sengkeli, Kg. Kara, Belaga, Lawas, Lambir National Park in Miri, Mount Serapi, Mount Dulit, Hose Mts, Bukit Lumut, Sugai Lemanak, Balleh, Gunung Pueh Forest Reserve, Kota Forest Reserve in Lawas and Mount Santubong. It can be found from 100 m altitude up to 1500 m. It grows in heath forest, beach vegetation and montane mossy forest. It is different from the other species in having hirsute hairs on almost parts of the plants.

Nepenthes lowii It is found widespread in montane forest of the northeastern part of the state, which includes Mt. Murud, Mt. Mulu, Gunung Api, Bukit Lawai; it has been collected only on Bukit Temedu in western part of the state. It is a highland species occurring from 900 m to 2200 m altitude. It is a common climber of mossy forest but may also be epiphytic on tree; it is found scrambling in ericaceous scrub of montane vegetation. It is similar to *N. ephippiata* in having numerous bristles on lower surface of pitcher's lid but differs in having inconspicuous peristome rim and ribs of the upper pitcher.

Nepenthes muluensis The author has collected this species from Mount Mulu and Mount Murud. It grows together with *Nepenthes tentaculata* in exposed scrub vegetation on the summit trail of both mountains from 1700 m to 2400 m altitude. It is related to *Nepenthes tentaculata* by it racemes inflorescence and simple flower pedicel, inner surface

of pitcher partly covered with exposed digestive glands. It differs from this species in having cylindrical stem, semi-amplexicaul leaf base, and marginal hairs on pitcher lid absent. These two species hybridized naturally on Mt. Mulu, forming a hybrid (Adam and Wilcock, 1992a)

Nepenthes northiana It has been collected from altitude 30 m to 200 m from several limestone hills in Bau Kuching District, which includes Seburan, Mount Tabai, Bukit Numpang, Bukit Kapor and Bidi Caves. It grows commonly in cervices overhanging semi-shaded vertical walls of limestone and gentle slopes in damp habitats.

Nepenthes pilosa It is a rare species in Sarawak. It has been collected once from Bukit Buli in Bario in mossy forest at 1600 m altitude. This species is very distinctive in having most parts of the plants densely covered with reddish-brown pilose hairs.

Nepenthes veitchii This species is recorded from Lawas Kayangeran Forest Reserve, Ulu Lawas Merapok, Ulu Tiang and Balleh, River Kenaba Upper Plieran, Bukit Berar, Ulu Sungai Kakus in Bintulu, Nanga Semparajah in Ulu Mujong, Mount Lesong, Mount Santubong, summit of Mount Lesong, Bukit Lumut in Ulu Mujong, Sungai Masia in Karangaran Forest reserve, between Mendalam and Terikan Rivers in Mulu, Mount Dulit, Ulu Melinau Fall, Batu Buli in Bario, Mount Mulu. It grows epiphytically on trees in low and high altitude mossy forests between 500 m to 1800 m. This species can easily be identified in the field by its epiphytic habit and unusual broad peristome of pitcher.

Species distributed outside Borneo

Nepenthes albomarginata This species differs from other species in the family by the presence of white velvety band around its pitcher mouth. It is occurring in Borneo, Peninsular Malaysia, Singapore and Sumatra. The distribution of the species is widespread and it has been collected from many parts of Sarawak which include Mt. Santubong, Loba Vebay Forest Reserve, Bukit Braag, Rantau Panjang, Teku, Bau (Bidi Cave, Bukit Jambusan, Bukit Jebong, Bukit Kapor), Bako Park (Telok Assam, Lakei Islands), Bawang, Mount Staar, Mount Bungoh, Mount Aping, Mount Matang, Mount Tiang Lake, Mount Mururong, Miri (Lambir Hill Summit). It is commonly in lowland area below 500 m altitude and sometimes can grow at higher altitude up to 1000 m. It grows in heath forest, along the coast on rock face, limestone vegetation, secondary scrub and mossy forest on low altitude mountain.

Nepenthes ampullaria This species is morphology related to *Nepenthes bicalcarata* by its paniculate inflorescence, urceolate lower pitcher, wholly glandular with overarched gland on pitcher inner surface but differs from the latter species in having very narrow deflexed pitcher lid, absence of two incurved thorns on the lid base and bracteolate flower pedicels. This is a common species in Sarawak; it has been from Bako Park, Sungai Dua Baram, Pulau Bruit, Kayangkeran Forest Reserve, Kelapaan, Sungai Raya, Selalang Forest Reserve, Bawang, Bau, Tebuan Hilir Kuching, Sungai Tutus, and Sebanding Forest Reserve. It is a lowland species, commonly grows below 100 m altitude but may grow up to 600 m altitude. It is a common plant on roadside clearing, heath forest, margin of secondary swamp vegetation, peat swamp forest, c sometimes found within the gap of tall canopy lowland dipterocarp forest.

Nepenthes gracilis This species occurs in most part of the state, which includes Batang Baram, Bau, Batu Kawa, Bako Park, Bawang, Betong-Saribas Forest Reseve, Bukit Sampadan Kuching, Lambir Miri, Lundu, Marudi, Matang Road Kuching, Melugu, Mount Matang and Sungai Rayu. It is a lowland species, commonly found below 500 m altitude but may occurs up to 1200 m altitude. This species is closely related to *Nepenthes reinwardtiana* by its triangular stem, winged leaf base, pitcher shape and partly glandular pitchers but differs in having racemes inflorescence, simple flower pedicels, inner pitcher cavity covered with exposed digestive glands, lower lid scarcely covered with nectar glands and the absence of two big black spots on the pruiose zone on the inner cavity surface of upper and lower pitcher.

Nepenthes mirabilis It grows extensively in damp habitats or swampy areas, fringes of swampy vegetation, in secondary vegetation and on roadside clearing. It is one of the

common lowland species encountered in Sarawak; it has been collected from Bawang (*N. mirabilis* var. *biflora* Adam and Wilcock, 1992b), Kuching area, Bau area, Lundu, Betong, Sg. Rayu, Pedawan, Wau, Balulo and Sematan. It is found growing from sea level to 500 m and rarely up to 1000 m altitude.

Nepenthes rafflesiana The distribution of the species in Sarawak is widespread. It has been collected from Matang, Lobok Pasar, Batang Baram, Sungai Bintawa, Rantau Panjang, Teku Road, Lundu, Kuching, Bako, Bukit Tambi, Baram, Lambir Hill, Bukit Lumut, Carapa Lop in Ulu Mujong Balleh and Mt. Aping. It can be found from sea level up to 1000 m altitude. It grows in heath forest, secondary vegetation on roadside clearing, low altitude mossy forest, and gap of dipterocarp forest, peat swamp forest and Padang keruntum forest.

Nepenthes reinwardtiana This species has been collected from Lawas Kayangeran Forest Reserve, Pa' Ukat Bario, Hose Mt., Nanga Pengiran in Balleh, Pelagus Rapid in Belaga, Bako National Park, Upper Rejang River, Sungai Bena in Kapit, Mount Dulit. It can be found commonly at low altitude and may be found up to 1300 m above sea level. It grows commonly on roadside vegetation or bare ground on steep or gentle slopes, growing epiphytically on trees, in montane mossy forest and coastal rock faces. It is related to *Nepenthes gracilis* by its sessile leaves, winged lamina base and triangular stem. It differs from other species by the presence of two black round spots on the waxy zone on the inner pitcher cavity.

Nepenthes tentaculata It is widely distributed on the mountain forest of Sarawak between 800 m to 2100 m altitude. It has been collected from Mount Berumput, Mount Mulu, Mount Api, Ulu Balleh, Mount Tibang, Hose Mts, Mount Dulit, Ulu Anap, Bukit Temedu, Mount Bungoh, Meruruon Plateau in Bintulu, Bukit Sekanjang, Mount Poi, Mount Santubong, Mount Matang, Bukit Sempadi in Kapit, Bukit Lawi, Mount Dulit, Mount Serapi and Mount Penrissen. It is a common species on the top half of Mount Murud; and this species has been recognized as *Nepenthes murudensis*. It is closely related to *N. muluensis* but differs in having triangular stem and winged lamina base.

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D-2

Purification and enzymatic characterization of an aspartic proteinase (Nepenthesin) from the insectivorous plant *Nepenthes distillatoria*

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E-1

Habitat conditions of *Utricularia spp.* at Lower-Silesian Province in Poland

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Carnivorous plants in Poland are represented among other by bladderworts (genus *Utricularia* – family *Lentibulariacae*). In Poland nowadays exist five species: *Utricularia vulgaris, U. neglecta, U. minor, U. intermedia, U. ochroleuca.* Sixth species *U. Bremii* was present tens of years ago, which does not exists now and needs cataloguing. Number of sites of *Utricularia* spp. is different. Common plant is *U. vulgaris* but *U. ochroleuca* is the rarest species ("Vulnerable" species V – taxa believed likely to move into the E – "Eendangered" category in the near future if the causal factors continue operating [Zarzycki *et al.*, 1992]). Remaining species are rare. Number of undiversified sites of *Utricularia* species according to ATPOL Distribution Atlas (Hermanowicz *et al.*, 1999) as follow: in Poland (312685 km^2) - *U. vulgaris* 835, *U. minor* 349, *U. intermedia* 314, *U. neglecta* 185, *U. ochroleuca* 21, at Lower-Silesian Province (19948 km^2) - *U. vulgaris* 40, *U. minor* 19, *U. intermedia* 11, *U. neglecta* 15, *U. ochroleuca* 3. Number of uncertain sites is: Poland - *U. ochroleuca* 3, Lower-Silesian Province - none.

Ecological studies of water plants (habitat conditions, ecological constitution of species and ecological organization of their populations) determine important circumstances for their active protection. This type of studies need bladderworts.

The aim of this study was to present the differentiation of habitats of bladderworts in respect of chemical features, whether habitats of different species show similarity in relation to oneself. Seventeen sites were chosen at Lower-Silesian Province (Poland). The contents of NO^{-}_{2}, NO^{-}_{3}, NH^{+}_{4}, PO^{-2}_{4}, K^{+}, Ca^{+2}, Mg^{+2}, Na^{+}, Fe^{+3}, SO^{-2}_{4}, hardness of water, humus acids and pH have been examined in the water samples. It has been stated, that sites for species are differed. This differentiation within of species is slight. Greater differentiation is between species. Investigated waters are characterized by different trophic status. They are mesotrophic, in mosts cases eutrophic, more seldom dystrophic.

It has been stated, that *U. vulgaris, U. neglecta* and *U. ochroleuca* exist in eutrophic waters. But *U. minor* and *U. intermedia* prefer dystrophic ones, which are characterized particularly by lower pH and higher content of humus acids. On the basis of the results using method of cluster analysis (Legendre and Legendre, 1998; StatSoft, 2001) tree diagram was constructed (Legendre and Legendre, 1998; StatSoft, 2001). Similarity of sites has been determined. Tree diagram shows least similarity of *U. minor* and *U. intermedia* sites in relation to the sites of remaining species of *Utricularia*

Eutrophication and also specific chemical pollution causes stress and can be vital significance for these species. Whole populations of sensitive species could be destroyed. The results of this type of investigations should be taken into account in active protection of plants and their biotopes.

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E-2

Evaluation of inter-simple sequence repeat (ISSR) for systematic relationship of some terresetrial species of *Utricularia* L. (Lentibulariaceae)

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Abstract. Inter-simple sequence repeat (ISSR) amplification was evaluated for its applicability as a genetic marker system to establish relationships among ten terrestrial *Utricularia* species. The resultant products were subjected to agarose gel electrophoresis and the banding patterns were compared among *Utricularia alpina*, *U. amethystina*, *U. bifida*, *U. caerulea*, *U. calycifida*, *U. humboldtii*, *U. longifolia*, *U. praelonga*, *U. triflora* and *U. uliginosa*. ISSR amplification generated multiple banding profiles with the six primers from all DNA samples, with an average of 39.3 fragments per primer. A total of 236 bands were detected by six primers. Primer UBC 889 presented the highest number of bands while primer UBC 891 produced the least number. Among the species studied *U. alpina* and *U. longifolia* were found to be most closely related. *Utricularia bifida* and *U. humbodtii* stand the second position in term of similarity whereas *U. cearulea* and *U. triflora* were placed most far from the other species. The genetic relationships of the species estimated by ISSR markers indicate the reliability of ISSR markers for estimation of genotypes. The characteristics feature of ISSR markers (*i.e.*, polymorphism, generation of information and ease of handling) suggest their applicability in molecular systematic studies.

Key words: ISSR, Lentibulariaceae, systematic relationship, terrestrial *Utricularia*

Introduction

Utricularia, a member of the family Lentibulariaceae encompasses about 214 species, is distributed throughout the world except for nival and polar ice zones (Taylor, 1989). Among the carnivorous plants *Utricularia* is unique because of the structural complexity of its traps, thought to be the most intricate in the plant kingdom and the rapid movement of the opening and closing of its trapdoors (Meyers and Strickler, 1979). They have pioneered many habitats including wet grounds, ponds, lakes and other marshy areas, epiphytic conditions and seasonal deserts.

Since *Utricularia* was first reported in "Species Plantarum" where Linnaeus (1753) listed only seven species, it has received considerable attention from many taxonomists. Many important account from different parts of the worlds mainly based on morphology and floristics were produced in the 20th century. For instance, Perrier (1955) paid an attention on the Malagasy species of *Utricularia* while Fernandez-Perez (1964) worked out with Colombian species. The species from Argentina were described

by Dawson (1973). Fromm-Trinta (1972) was successful in producing an account for the Brazilian species of *Utricularia* and Thor (1988) has sorted out the Scandinavian species. In Japan, systematic studies on the Lentibulariaceae was covered by Komiya (1972). Komiya (1973) proposed a new sub-division of the Lentibulariaceae. Komiya and Shibata (1980) showed the distribution of the Japanese Lentibulariaceae. Subramanyum (1979) studied the Indian *Utricularia* intensively. Recently Crow (1992) dealt with *Utricularia* of Costa Rica. However, the most significant systematic work is the monograph of *Utricularia* produced by Taylor (1989). *Utricularia* also received cytological as well as palynological investigation. Casper and Manitz (1975) were most successful in counting chromosome for European species and Kondo (1966, 1973) for non European species. Cytology of Indian species were conducted by Subramanyam and Kamble (1968). The pollens of the *Utricularia* were investigated by Thanikaimoni (1966), Huynh (1968) and Sohma (1975a, b).

Simple sequence repeat (SSRs or microsattelite) consists of tandem arrays of short oligonuleotide sequences 2-6 bases in length. Throughout the eukaryotic genome SSRs are very common (Tautz and Renz, 1984) and highly polymorphic in length (Levinson and Gutman, 1987). Inter-simple sequence repeats (ISSRs) are a new type of DNA marker which are generated from a single primer PCR and the primer is designed from a microsattelite motif. ISSR involves the use of microsattelite sequences directly in the polymerase chain reaction (PCR) for DNA amplifications (Gupta *et al.*, 1994). ISSR primers contain sequences complementary to a SSR motif and also a 1-3 base anchor at either the 3′ or 5′ end. Base positions within the anchor may contain any nucleotide other than the needed to continue the repeat sequence. This technique enables amplification of genomic DNA and provides information about many loci simultaneously. The utility of this approach stems from its simplicity and reproducibility, the higher number of polymorphism revealed and the fact that there is no prior need for DNA sequence information from the organism under study. Zietkiewicz *et al.* (1994) first used this approach to distinguish between closely related plant genotypes. This technique has successfully employed for cultivar identification (Farnandez *et al.*, 2002; Pasqualone *et al.*, 2001; Prevost and Wilkinson, 1999), population study (Crawford *et al.*, 2001; Lai *et al.*, 2001; Qiam and Hong, 2001; Zhou *et al.*, 1999) and interspecific relationships (Huang and Sun, 2000; Joshi *et al.*, 2000; Wolfe and Randle, 2001; Xu and Sun, 2001).

We used ISSR markers first to assess the distinctness of ten terrestrial species of *Utricularia*. The present study is the first report of the applicability of the nuclear DNA marker ISSR-PCR in characterizing genetic affinities at the interspecies level in the genus *Utricularia*. The primary objectives are to establish a baseline of systematic relationships for the *Utricularia* species and to test the utility of ISSR markers in estimating relationships among the species. Our results permit us to establish genetic relationships among ten terrestrial species within the genus *Utricularia*, and show that the ISSR approach is an interesting tool for plant molecular systematics.

Materials and Methods

Plant material *Utricularia alpina* Jacq., *U. amethystina* Salzm. *ex* A. St. Hil., *U. bifida* L, *U. caerulea* L, *U. calycifida* Benj., *U. humboldtii* Schomb., *U. longifolia* Gardner, *U. praelonga* A.St.Hil. *et* Girard, *U. triflora* P. Taylor and *U. uliginosa* Vahl were analyzed in this study. Fresh leaves were collected from plants from *in vitro* culture under long-term conservation conditions at the Laboratory of Plant Chromosome and Gene Stock, Graduate School of Science, Hiroshima University, Japan.

DNA extraction Total genomic DNA was extracted from the leaves following Kawahara *et al.* (1995) with slight modification. 1.0-1.8g of leaf was homogenized in a mortar using liquid nitrogen. The powdered tissue was transferred to a 20ml capacity of capped, sterilized centrifuge tube containing 10ml of wash buffer [0.1M Tris-HCl at pH

8.0, 2% 2-Mercaptoethanol (w/v), 1% Polyvinylpyrolidone K-30 (w/v), 0.05M L-Ascorbic acid, dissolved in distilled water]. After shaking gently for 10 min the tube was centrifuged 10,000 rpm at 20℃ for 10 min. The supernatant was discarded from the tube and this process was repeated until the solution becomes transparent. After removing the supernatant, 10ml of CTAB buffer [2% Cetyltrimethylammonium bromide (CTAB; w/v), 1.4M NaCl, 0.1% Tris-HCl at pH 8.0 (w/v), 20mM EDTA-Na2, dissolved in distilled water] and 0.5ml 2-Mercaptoethanol were added to the tube followed by an incubation at 55℃ for 60-90 min with a view to supply the stabilization of DNA. Following that 10ml chloroform:isoamylalcohol (24:1 v/v) was added to the tube and was shaken gently for 10 min and centrifuged at 10,000 rpm for 10 min. The supernatant was transferred to a new sterilized centrifuge tube and it was continued until there was no precipitation on the border of the supernatant layer and chloroform:isoamylalcohol layer. The final supernatant was transferred to an another centrifuged tube and 10ml of 2-propanol was added followed by a centrifugation at 10,000 rpm for 15 min at 4℃. After discarding the solution 5ml 70% chilled ethanol was added to wash the pellet and was centrifuged at 10,000 rpm for 5 min. DNA was dried after decanting the ethanol and the dried DNA pellet was dissolved in 450μl TE solution (10mM Tris-HCl and 1mM EDTA) with 0.1mg/ml RNase (Sigma). After incubation for 1 hour at 37℃ the solution was transferred in a 1.5ml sterilized ependorf tube. 250μl of neutral equilibrated phenol and 250μl of chloroform:isoamyl alcohol (24:1) were added to the tube. After shaking for 10 min the tube was centrifuged for 10 min. The upper phase was transferred to a new tube. 500μl chloroform:isoamyl alcohol was added and was centrifuged for 10 min. The upper aquous solution was transferred to a new tube. Then 50μl of 3M sodium acetate and 500μl of 99.5% chilled ethanol were placed in the tube and they were kept at –80℃ for 20 min. The DNA pellet was found by spinning in a microcentrifuge at 15,000 rpm for 15 min at 4℃. The supernatant was removed and the pellet was washed with 70% chilled ethanol using centrifugation at 15,000 rpm for 15 min at 4℃. The ethanol was discarded and the DNA was dried into a Halogen Vacuum Concentrator for 3-5 min. The isolated DNA was dissolved in TE buffer and stored at –20℃.

ISSR primers One hundred ISSR primers (primer set # 9) were purchased from the University of British Columbia, Biotechnology laboratory (UBC, Vancouver, Canada). These primers were 15-mer to 22-mer and many of them consisted of di-, tri-, tetra-, and pentanucleotide repeat motifs of which dinucleotide repeats were with anchor. Out of 100 primes procured, a total of 72 primers were screened for PCR amplification. Finally, we selected 6 primers (Table 1), by their number and consistency of amplified fragments, for analyzing ten species of *Utricularia*.

PCR amplification and Electrophoresis DNA amplification were performed in a 10μl reaction volume containing 10ng of template DNA, 1μM of a single primer (UBC, Vancouver, Canada), 1μl of x10 Taq buffer, 0.8μl of dNTP mixture and 0.05μl of Taq polymerase enzyme. The reaction mixture was overlaid with 30μl mineral oil. Amplifications were performed in a PTC-100 thermal cycler programmed for an initial step of 5 min at 94 ℃, followed by 35 cycles of 1m at 94℃, 45s at 50℃ and 2 min at 72℃, and a final 5 min extension at 72℃.

ISSR bands were characterized on 1.5% agarose gels in 1X TAE buffer by loading the entire reaction volumes into prepared wells. Gels were run until a bromophenol blue indicator dye ran morethan 70% from the well. The gels were stained in ethidium bromide and bands were visualized in UV light. The molecular size of the fragments was estimated by reference to a 1kb ladder (Pharmacia).

Data analysis The banding pattern obtained using six ISSR primers were analyzed to estimate genetic relationships among the ten terrestrial *Utricularia* species. The ISSR bands were interpreted as dominant markers and were scored as diallelic characters either as 1 (present) or 0 (absent). The data matrices were analyzed by the SIMQUAL program of the NTSYS-pc version 2.1 (Rohlf, 2000). A pair-wise similarity matrix was calculated for a measure of within species similarities using the simple matching coefficient. This

similarity matrix was employed to construct a dendrogram by the unweighted pair group method with arithmetical averages (UPGMA), using the SAHN-clustering and TREE programs from the NTSYS-pc, version 2.1 package (Rohlf, 2000). A pair-wise dissimilarity matrix was also made using the DIST coefficient.

Results and Discussion

ISSR analysis ISSR amplification using six primers produced a total of 236 fragments from the ten *Utricularia* species. The total number of bands generated six primers in the ten species varied from 18 in *Utricularia amethystina*, *U. humboldtii* and *U. uliginosa* to32 in *U. longifolia*. Figures 1, 2, 3 and 4 represent the amplification patterns generated using primer UBC 842, UBC 888, UBC 889 and UBC 890 across the ten species. The least and highest number of fragments generated per primer in all species studied were 33 (primer UBC 888 and UBC 891) and 48 (primer UBC 889), respectively (Table 1). The number of fragments produced by other primers ranged from 37 (primer UBC 864) to 43 (primer UBC 890). The average number fragments produced by all analysed primers is 39.3.

Genetic relationships among *Utricularia* species

The ISSR marker was found efficient in the study of the genus *Utricularia*, where genetic characterizations of the nuclear genomes are almost lacking. We used the ISSR technique to analyse ten terrestrial *Utricularia* species with a view to determine their genetic relatedness. According to our findings, *Utricularia* species could be characterized using ISSR markers. The estimated similarity coefficient among ten species varied from 5.82 (*U. caerulae*/ *U. longifolia*) to 8.66 (*U. alpina*/ *U. longifolia*).(Table 2). The dendrogram erected by the UPGMA method using 236 ISSR markers scored in the ten species presents 2 defined groups, Group I and Group II. (Fig. 5).

Group I bears the species *U. alpina* and *U. longifolia*. The highest similarity value was found in this group among all taxa studied at a similarity level of 8.6 (Table 2). The dendrogram shows that *U. alpina* and *U. longifolia* seem to be nearest. In contrast, *U. caerulea* show the highest dissimilarity value with *U. longifolia* (Table 3).

Group II forms the cluster between *U. amethystina* and *U. calycifida*; *U. bifida* and *U. humboldtii* ; *U. praelonga* and *U. uliginosa*. The cluster consisting of *U. bifida* and *U. humboldii* also exhibits a high level of similarity (7.63) followed by the cluster containing *U. amethystina* and *U. calycifida* (7.48). In this group the cluster between *Utricularia amethystina* and *U. calycifida* is joined with the cluster *U. bifida* and *U. humblodtii* to which another cluster containing *U. praelonga* and *U. uliginosa* is joined followed by *U. caerulea* while *U. triflora* is the last taxon.

The Lentibulariaceae have not been thoroughly investigated from the molecular point of view although some other carnivorous plant families were investigated using the molecular tools including Droseraceae (Albert *et al.*, 1992) and Saraceniacee (Bayer *et al.*, 1996). Many authors paid an attention on the largest carnivorous genus *Utricularia* based on morphology (Taylor, 1989; Crow, 1992), palynology (Huynh, 1968), and cytology (Casper and Manitz, 1975; Kondo, 1973; Subramanyum and Kamble, 1968). This unique genus, however, did not receive any attention so far from ISSR or other molecular markers. Recently an isozyme study was carried out only for two infraspecific taxa of the aquatic *U. australis* (Araki, 2000).

Our results indicate that the species *Utricularia alpina* and *U. longifolia* show the highest affinity among the species studied. This might be due to sharing a considerable number of markers between these two species which indicates that *U. alpina* and *U. longifolia* form a close genetic assemblage. This result support the morphology of these two species because there share the characteristics by elliptic or obovate leaves, curved filament, ovoid ovary and ovate to ovate-deltoid calyx lobes. However, Taylor

(1989) placed these two species in two different sections. To have the better resolution, some other molecular tools as well as large number of sampling are necessary to be conducted. From the cytological point of view, it is impossible to compare between these species because chromosome number is reported in *U. alpina* (Kondo, 1966) while chromosomal information is missing in *U. longifolia*.

Group II bearing eight species of *Utricularia* forms three subclades including *U. amethystina* with *U. calycifida*; *U. bifida* with *U. humboldtii* and *U. praelonga* with *U. uliginosa*. (Fig. 5). *Utricularia amethystina* form clade with *U. calycifida* showing the close relationship to each other. They are morphologically in the same line by having unequal calyx lobes, globose capsule and obliquely ovoid seeds. In *U. bifida* and *U. humboldtii* some characteristics are found to be similar including subulate spur, distinct anther thecae, basifixed bracts and linear or linear to subulate bracteoles which is supported by our analysis. Although palynlogical information is available (Huynh, 1968), however, our data does not match with that. None of these species was cobered by cytological data. The last clade in Group II comprise the species *U. praelonga* and *U. uliginosa* and these are sharing ovate bracts, short style and subulate or narrowly linear bracteoles.

Utricularia caerulea and *U. triflora* are far away from the other species showing the lower values of similarity with respect to the rest of the species, and this can be explained by the fact is that these taxa present the most distant genetic relationship compared to other species studied.

Out of ten species studied, chromosome numbers have been counted only in *Utricularia alpina* (Kondo 1966 as '*Orchyllium alpinum*'), *U. caerulea* (Kondo, 1973 as '*U. recemosa*') and *U. uliginosa* (Tanaka and Uchiyama, 1988 as '*U. yakusimensis*'). Since the chromosome information is very much lacking and fragmentary therefore our results can not be compared with cytological investigation properly.

In this study we did not compare *Utricularia* with an outgroup species because the aim of this investigation was to establish interspecific relationships rather than phylogenetic relationships. Although we included only ten species in this study, but we are currently evaluating ISSR markers for other species of *Utricularia* as well. Using these efficient markers we will be able to figure out the phylogenetic relationship and evolution of the unique carnivorous genus *Utricularia*.

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Table 1 . List of primer sequences used along with the scorable fragments resulting from each primer

Primer code	Sequencesa	Repeat	No. of fragments scored
UBC 842	GAG AGA GAG AGA GAG AYG	(GA)8YG	42
UBC 864	ATG ATG ATG ATG ATG ATG	(ATG)6	37
UBC 888	BDB CAC ACA CAC ACA CA	(CA)7BDB	33
UBC 889	DBD ACA CAC ACA CAC AC	(AC)7DBD	48
UBC 890	VHV GTG TGT GTG TGT GT	(GT)7VHV	43
UBC 891	HVH TGT GTG TGT GTG TG	(TG)7HVH	33

a Y stands for pyrimidine, B for non-A, D for non-C, V for non-T and H for non-G residues

Table 2 : Similarity matrix among ten species of *Utricularia* using the simple matching coefficient based on 236 ISSR fragments scored

Species	*U. alpina*	*U. amethystina*	*U. bifida*	*U. caerulea*	*U. calycifida*	*U. humboldtii*	*U. longifolia*	*U. praelonga*	*U. triflora*	*U. uliginosa*
U. alpina	1									
U. amethystina	7.08	1								
U. bifida	6.61	7.16	1							
U. caerulea	6.22	7.08	7.08	1						
U. calycifida	6.14	7.48	7.16	6.61	1					
U. humboldtii	6.77	7.48	7.63	7.24	7.32	1				
U. longifolia	8.66	6.53	6.37	5.82	6.06	6.85	1			
U. praelonga	6.53	7.08	7.08	6.85	6.61	7.08	6.29	1		
U. triflora	6.53	7.08	6.61	6.85	6.92	6.92	6.14	6.22	1	
U. uliginosa	6.77	7.16	7.01	6.61	7.16	7.16	6.53	7.4	6.29	1

Table 3 : Dissimilarity matrix of ten species of *Utricularia* using DIST coefficient based on 236 ISSR fragments

Species	*U. alpina*	*U. amethystin.*	*U. bifida*	*U. caerulea*	*U. calycifida*	*U. humboldti*	*U. longifolia*	*U. praelonga*	*U. triflora*	*U. uliginosa*
U. alpina	0									
U. amethystina	5.39	0								
U. bifida	5.81	5.32	0							
U. caerulea	6.14	5.39	5.39	0						
U. calycifida	6.21	5.01	5.32	5.81	0					
U. humboldtii	5.68	5.01	4.86	5.24	5.17	0				
U. longifolia	3.65	5.88	6.01	6.46	6.27	5.61	0			
U. praelonga	5.88	5.39	5.39	5.61	5.81	5.39	6.08	0		
U. triflora	5.88	5.39	5.81	5.61	5.54	5.54	6.21	6.14	0	
U. uliginosa	5.68	5.32	5.47	5.81	5.32	5.32	5.88	5.09	6.08	0

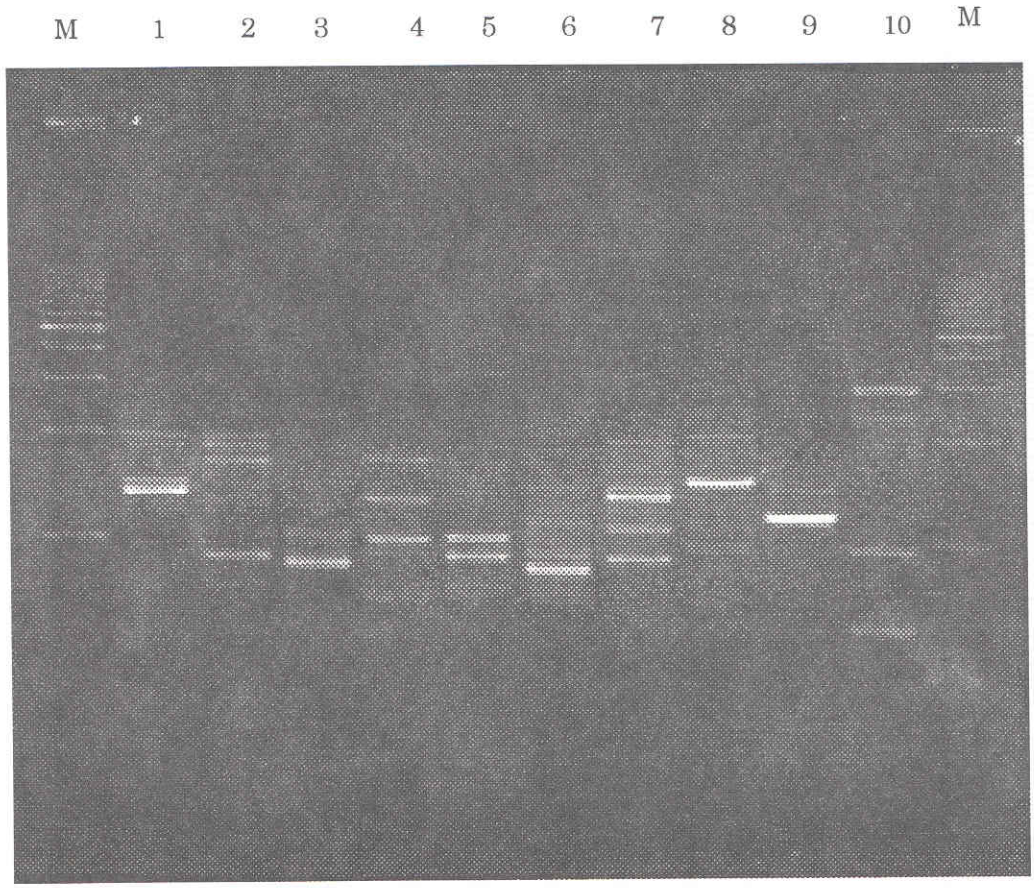

Figure 1 : Inter-simple sequence repeat (ISSR) banding profiles obtained on 1.5% agarose gel for ten *Utricularia* species with the primer UBC 842. M = Molecular size marker (1 kb), 1 = *U. alpina*, 2 = *U. amethystina*, 3 = *U. bifida*, 4= *U. caerulea*, 5 = *U. calycifida*, 6 = *U. humboldtii*, 7 = *U. longifolia*, 8 = *U. praelonga*, 9 = *U. triflora*, 10 = *U. uliginosa*

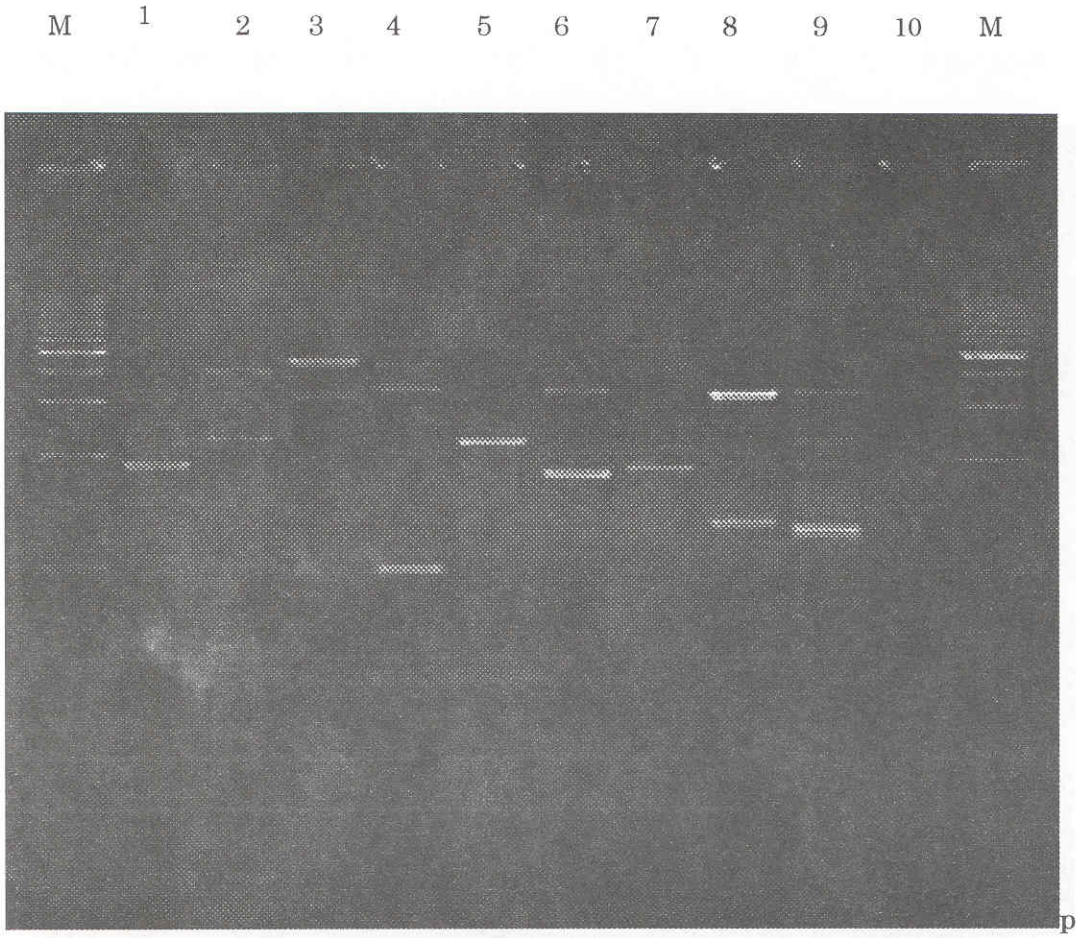

Figure 2 : Inter-simple sequence repeat (ISSR) banding profiles obtained on 1.5% agarose gel for ten *Utricularia* species with the primer UBC 888. M = Molecular size marker (1 kb), 1 = *U. alpina*, 2 = *U. amethystina*, 3 = *U. bifida*, 4= *U. caerulea*, 5 = *U. calycifida*, 6 = *U. humboldtii*, 7 = *U. longifolia*, 8 = *U. praelonga*, 9 = *U. triflora*, 10 = *U. uliginosa*

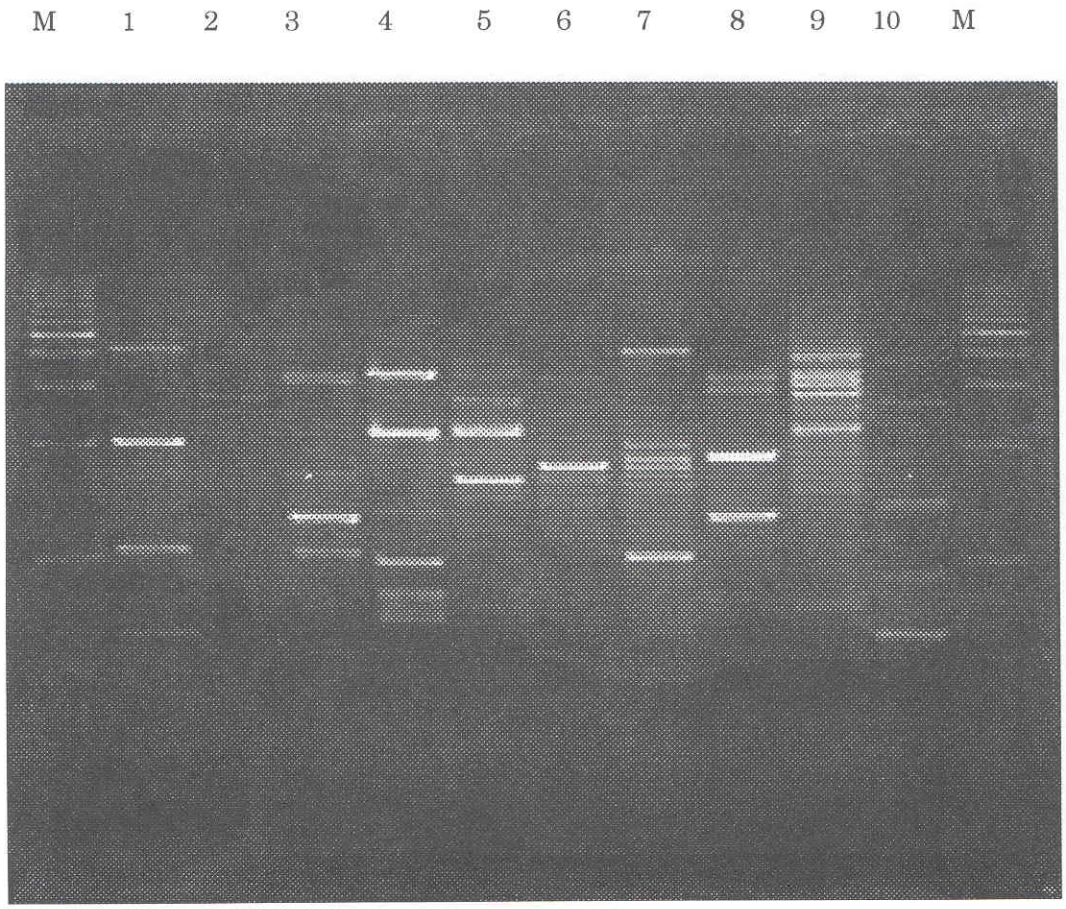

Figure 3 : Inter-simple sequence repeat (ISSR) banding profiles obtained on 1.5% agarose gel for ten *Utricularia* species with the primer UBC 889. M = Molecular size marker (1 kb), 1 = *U. alpina*, 2 = *U. amethystina*, 3 = *U. bifida*, 4= *U. caerulea*, 5 = *U. calycifida*, 6 = *U. humboldtii*, 7 = *U. longifolia*, 8 = *U. praelonga*, 9 = *U. triflora*, 10 = *U. uliginosa*

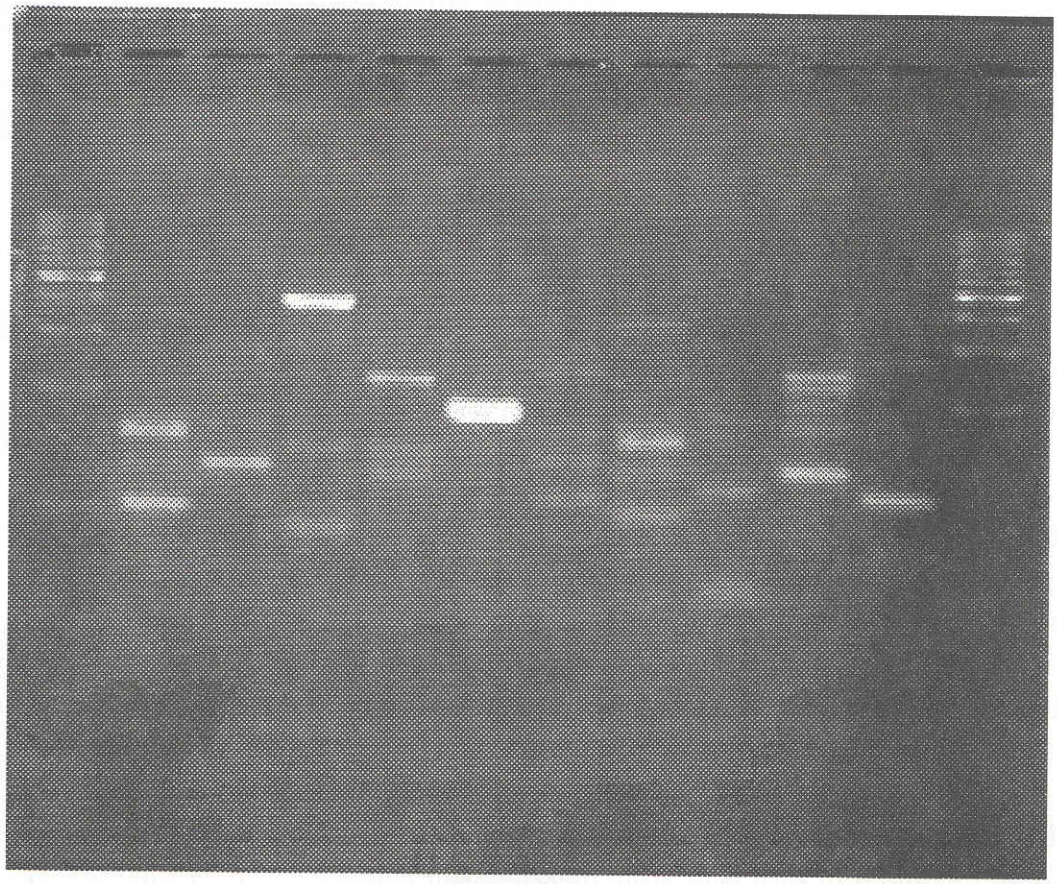

Figure 4 : Inter-simple sequence repeat (ISSR) banding profiles obtained on 1.5% agarose gel for ten *Utricularia* species with the primer UBC 890. M = Molecular size marker (1 kb), 1 = *U. alpina*, 2 = *U. amethystina*, 3 = *U. bifida*, 4= *U. caerulea*, 5 = *U. calycifida*, 6 = *U. humboldtii*, 7 = *U. longifolia*, 8 = *U. praelonga*, 9 = *U. triflora*, 10 = *U. uliginosa*

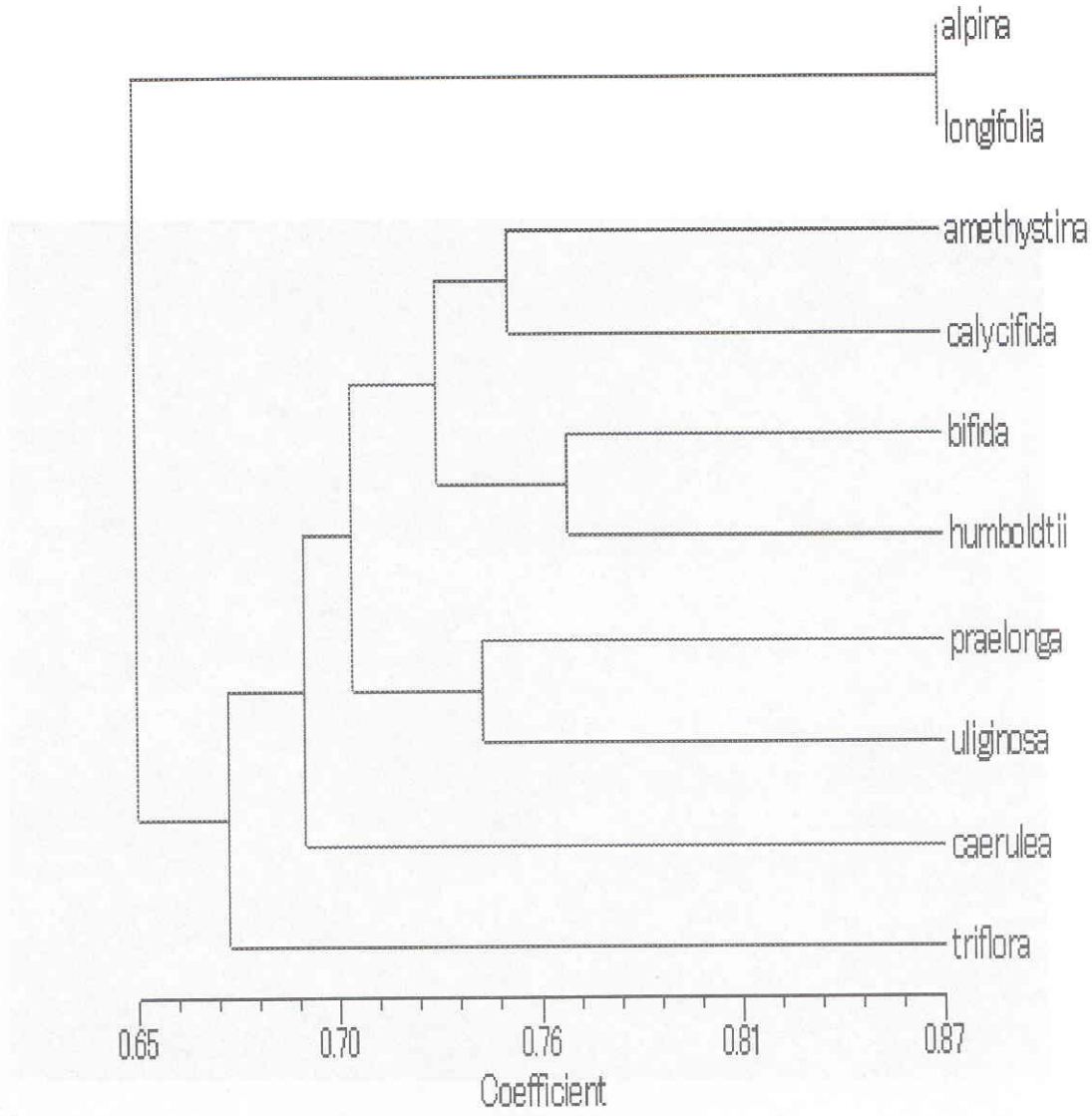

Figure 5 : Dendrogram showing the genetic relationships among ten terrestrial *Utricularia* species constructed by the UPGMA cluster analysis, using simple matching coefficient based on 236 ISSR fragments

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E-3

Utricularia macrorhiza Le Conte in Japan

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Introduction

Utricularia macrorhiza Le Conte was described as a species distributing in middle to north of North America (Ann. Lyc. New York 1:73,t.6,fig.2, 1824). In Asia this species is widely distributed in NE-Siberia, Kamtchatka, Sakhalin, Amur and Ussuri regions in Russia and westwards to Altai, Mongolia and northern China (Rebristaja *et al.*, 1983; Taylor, 1989; Takahashi *et al.*, 1994). We found that this species is also distributed in Japan .

***Utricularia macrorhiza* in Japan** M.Toyama collected a specimen in Toyokoro Town, Tokachi District, Hokkaido on August 10, 1988 for the first time. Komiya and Shibata identified it as *Utricularia macrorhiza* regarding the floral structures on August 27, 1992. Prior to that, Komiya and Shibata collected another specimen quite similar to the first specimen in Kizukuri Town, Nishitsugaru-Gun, Aomori Prefecture in the northern part of Honshu on July 17, 1991 and identified it as *U. macrorhiza.* They also confirmed further distribution in Akita Prefecture on October 21, 1997.

Comparison and confirmation of specimens of the species collected in Japan and the U.S. Komiya and his group made field trips to northern California, Oregon, Washington, Alaska, North Carolina and Florida in the U.S. in 2000 and 2001 and collected several specimens very similar to each other and to the above specimens. They are all determined to be placed within *U. macrorhiza.*

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Figure legends

Figure 1. A specimen of *Utricularia macrorhiza* collected in Toyokoro Town, Hokkaido by Komiya and Shibata (August 27, 1992; NDC-4455)

Figure 2. Distribution map of *Utricularia macrorhiza* in Japan

Photograph 1. A huge bladderwort, *Utricularia macrorhiza* collected in Toyokoro Town, Hokkaido on August 27, 1992.

Photograph 2. Habitat of *U. macrorhiza* in Kizukuri Town, Aomori Prefecture on August 30, 1994.

Photograph 3. Flower stalks with flowers and fruits of *U. macrorhiza*.

Photograph 4. Close-up of the flowers of *U. macrorhiza*.

Photograph 5. Turions of *U. macrorhiza.*

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F-1

Conservation and management of *Drosera indica* red flowered form and its natural habitat as the natural monument autholized by the Aichi Prefectural Government

Tsunenori Asai

Toyoake City Protection Committee of Cultural Properties

Abstract. Within the natural-monument protection area of *Drosera indica* authorized by the Aichi Prefectural Government, Toyoake City Protection Committee of Cultural Properties has been making progress in applied ecology for environmental improvement in natural habitat and conservation of the species on long-term basis and collecting continuously information on conservation of the species in literature. The protection area can be open to public by putting time limit for the purpose of education on conservation of nature and natural resources and study for life.

Toyoake City grows *Drosera indica* red-colored flower form as the natural monument authorized by the Aichi Prefectural Government. This local population of the species is well isolated and does not have any gene flow from other populations. Toyoake City Protection Committee of Cultural Properties has been studying seed germination and developmental morphology, growth, microclimates, buffered and invaded plants and succession, carnivory or insect capture, herbivors, gene sequencing, plant demography and population biology and so on in *D. indica* to utilize better understanding and administration job for conservation and management on the long term base. On the other hand, the Committee has been exchanging information on the species conservation by collecting literatures, attending study and training courses, practical and observation visitation to other protected areas to learn better comprehensive administration on environmental adjustment, conservation of the species, full-seasons management of the area, education and training the volunteers and so on within the protection area. Additionally, the Committee has organized open days to the protected area to observe live *D. indica* and public lecture courses, released some informations on the protected area to media such as TV, newspapers, public bulletins, and so on, and published and distributed some leaflets on the natural monument *D. indica*, for the purposes to become well-known and recognized protection-area by using education and advertisement sources. Concerning biological aspect, public people should visit the protectead area of *D. indica* as the natural monument during the period of dates open to public and observe carefully plants of the species by face close-up to plants and learn how much important the natural resources of wild plants are. *Drosera indica* is very useful medium for conservation education as well as life education. As the conclusion, management of the protected area opening to public and plants of *D. indica*, fruits and failure by present, future further plans and activities are described in the poster.

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F-2

Plant community and carnivorous plants of Icchoda Bog

Mikio Suzuki and Conservationist group for the Icchouda Bog

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Abstract. Conservation issue and activity for the Icchouda Bog , where the plant community including several carnivorous plant species of boggy area have been preserving as the natural monument authorized by Aichi Prefecture, Japan, since 1984, are introduced.

The Icchouda bog is located at the northwestern part of Taketoyo-Cho, altitude 40～50 m above the sea-level, the base of Chita Peninsula, south of Nagoya City in Aichi Prefecture. At the beginning of their land reclamation of their land area of 89 ha for land development for the purpose of agricultural utilization in 1982, the boggy area of 11,000 m^2 was chosen and put up a fence round for wildlife-protected area where people are not allowed to enter excepting they are permitted to enter and observe plants of wetland at their selected days in summer, late July till early September. The bog was authorized as a natural monument by Aichi Prefecture in March 1984. The bog is surrounded by forest vegetation dominated by *Pinus thunbergii* and *Eurya japonica* and has a fountainhead discharging continuouslu moderate acid water flow to maintain two bogs and wetland vegetation. The two bogs total 570 m^2 areas grow seven species of carnivorous plants including *Drosera indica* white colored flower form, rare and endangered to Japan, *D. spathulata* 'Kansai-type', *Utricularia nipponica*, endemic and scarcely endangered to Japan or scarcely endangered to Japan if the specimen is classified as *U. minutissima*, *U. bifida*, *U. racemosa* and *U. yakushimensis* as well as non-carnivorous *Eriocaulon nudicuspe* and so on and some rare insects such as a dragonfly of *Nannophya pygmaea*, that are all restricted to boggy areas and very rare flora and fauna in Japan.

The Icchouda Bog opens to public every summer season when the majority of the members of flora and fauna community reach commonly most active stage; e.g., open five different days from 9:30 am until 14:00 pm in this summer from late July to early September.

Members of the conservationist's group of the Icchouda Bog meet and work for the Bog once ever month to discuss on progress of guide programs, publishing newsletters, dispersing news media and so on about the Bog and education using the medium of the boggy plants and to have field maintenance works by pulling and removing weeds out, cutting too much grown twigs, stems, etc for succession control or adjustment and cleaning water ditch.

Their present activities involved with the natural monument Icchouda Bog are described and introduced.

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F-3

Growing *Aldrovanda vesiculosa*
Fourteen years experience of *in situ* and *ex situ* conservation

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Aldrovanda Cultivation and Conservation Club, Mitakaya Primary School; [1]Shingou No. 1 Primary School, Hanyu City, Saitama Prefecture, Japan

Abstract. Their conservation activities for *Aldrovanda vesiculosa* rinked with the national natural monument and its habitat at Hozouji Swamp, Hanyu City, that were awarded the 1989 Minister Prize of the Environmental Protection Agency of Japan, are introduced here.

Hanyu City, Saitama Prefecture, Japan has been preserving Japan's last locality of Aldrovanda vesiculosa and its habitat, although the other local populations of the species have already been all extinct last 50 years. The Hanyu City and its Board of Education and Hanyu City *Aldrovanda* Conservation Association have been conserving the species as the national natural monument authorized by the Japanese Ministry of Education, Science, Sports, Culture and Technology in 1966. Conservation project on *Aldrovanda vesiculosa* have awarded some funds for their purposes on conservation researches for newly development of conservation technology. Mitakaya Primary School located near by the reserved area of the habitat of the species established a club to learn how to conserve Aldrovanda for the purpose of educational utilization. In other words, they are very useful medium property of education.

The present display may explain history of their club, some episodes, highlights and present situation and problems in their activities on Aldrovanda conservation.

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F-4

Aldrovanda vesiculosa was rescued *in vitro* from extinction and is now conserved *ex-vitro*

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Abstract. *Aldrovanda vesiculosa*, an almost extinct aquatic carnivorous plant, was rescued for conservation by culture and propagation in vitro and is maintained ex-vitro through its whole life cycle.

Introduction

Since *Aldrovanda vesiculosa* was described by C. Darwin, it has attracted much academic interest and studies have been done widely on its morphology and mechanism of trapping *Daphnia* (Juniper *et al.*, 1989). However, because of recent deterioration of the environment, *Aldrovanda vesiculosa* is a species now close to extinction. The purpose of this research project is to rescue *Aldrovanda vesiculosa* in vitro from extinction and conserve it ex-vitro to conduct morphological and physiological research throughout the year.

Materials and Methods

Apical segments of *Aldrovanda vesiculosa* were excised and sterilized in 0.5% $NaClO_3$ for 5 min. After rinsing 3 times with sterilized DW, they were cultured in 1/10 MS liquid medium (Murashige and Skoog, 1962) at 25±2℃ under 16h light and 8h dark condition. After 1 month, developed axillary buds were excised from the plants for further subcultures. The procedure was repeated and the propagated plants were taken ex-vitro and kept outside in plastic containers with paddy field soil and tap water.

Results and Discussion

In vitro cultured *Aldrovanda vesiculosa* proliferated well, without trapping, by formation and development of axillary buds. The number of the plants doubled in 1 month (Fig.1). After transfer to ex-vitro, they kept growing in containers outside (Fig.2) trapping *Daphnia* (Fig.3, arrow), produced flower buds at the end of June (Fig.4, arrow), and flowered in July (Fig.5). Mature seeds harvested from the plants were sterilized in 0.5% $NaClO_3$ for 20 min and kept in 1/10 MS liquid medium. The seeds germinated in December (Fig.6) and the growth continued in vitro (Fig.7). The plants which were maintained outside produced winter resting buds in December (Fig.8), and germination was observed in the following spring (Fig.9). This study allowed us to conduct morphological and physiological research on *Aldrovanda vesiculosa* throughout a whole year. However, growth of *Aldrovanda vesiculosa* kept ex-vitro was often impeded

by proliferating algae to the extent that the plants eventually completely disappeared. The for this undesirable phenomenon needs to be sought out.

Acknowledgement

We would like to thank Board of Education of Hanyu City, the Saitama Aquarium in Hanyu City, and the members of *Aldrovanda vesiculosa* preservation group of Hanyu City for a gift of *Aldrovanda vesiculosa.*

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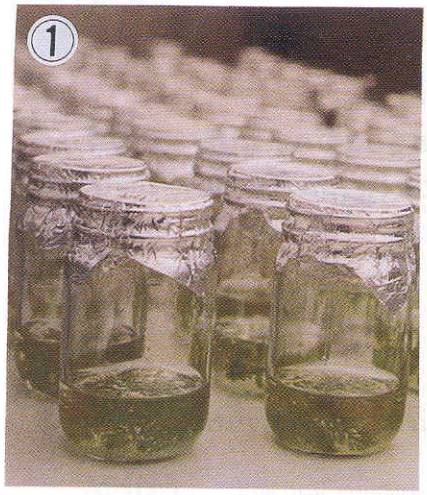
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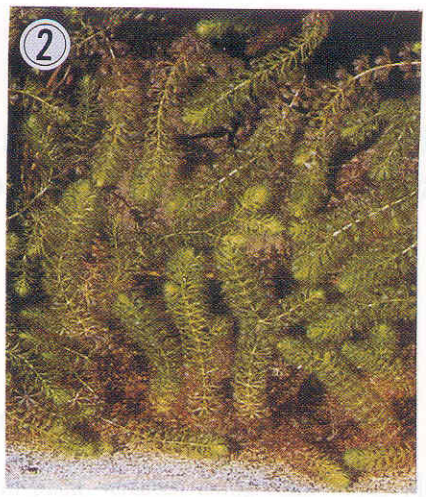
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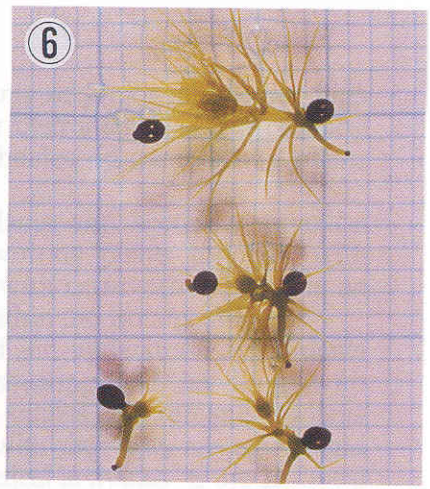
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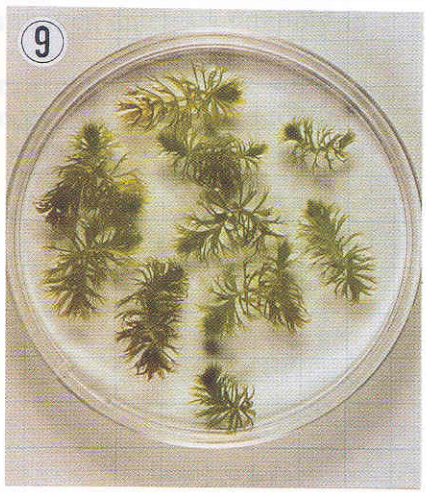
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F-5

Education and popularization to school children by plantation and practice of carnivorous plants in a program of Mito Municipal Botanical Park

Ayako Nishikawa

Mito Municipal Botanical Park, Mito City, Japan

Absract. Practical courses in plant sciences using carnivorous plants operated by Mito Municipal Botanical Park and their popularity are explained.

Numerous school chldren of primary schools and students of junior and senior high schools choose and spend the Botanical Park by themself for their integrated and practical learning in plant sciences. Among the programs, study themes on carnivorous plants are on their common favour.

We explain students firstly habitats of carnivorous plants and secondly practical manners of cultivation. Then, we let them plant carnivorous plants together with matches other than carnivorous plants. To select matches with carnivorous plants is an examination for them. Their potted plants are displayed within the Botanical Park.

Biology is obviously observation science and is mastered and understood by students practically by touching, smelling, cutting, smashing, dissecting and handling with observation by their own eyes. Our practical lessons allow school children and students can touch and learn how carnivorous plants capture preys with their trap structures and can plant them on pots to cultivate. Throughout their practical activities in the Botnical Park, they can learn structures, mechanisms, systems and moreover, interests not only in carnivorous plants but also in plants general.

Since we found carnivorous plants are excellent medium for the beginners such as school children and high school students to enter plant sciences, we would like to find, employ, and develop better methodologies more appropriate to meet biological aspect for education for them.

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G-1

Secretion of digestive enzymes in *Plumbago*

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Abstract. Phylogenetic studies, using molecular characters, indicate that *Plumbago* (Plumbaginaceae) is related to *Drosera* (Droseraceae) and Drosophyllum (Droserophyllaceae). Sepals of *Plumbago auriculata* Lam. and *Plumbago indica* L. bear large mucilage secreting trichomes, which resemble those of *Drosophyllum*. These trichomes are capable of capturing insects. We used the substrate film technique to test whether *Plumbago* trichomes can be stimulated to secrete proteolytic enzymes by the same stimuli that trigger secretion in *Drosera*. Several different stimuli were tested. There was strong secretion of proteases in response to 5 mM NaCl and NH_4Cl and a weaker response to 5 mM KCl. Yeast extract, which is highly stimulatory of protease secretion in *Drosera* fails to stimulate secretion in Plumbago. *Plumbago auriculata* and *Plumbago indica* are capable of secreting proteases in response to chemical stimulation but do not respond identically to all chemical stimuli effective with *Drosera*.

Introduction

Phylogenetic studies using molecular characters indicate that *Plumbago* (Plumbaginaceae) is related to *Drosera* (Droseraceae), *Drosophyllum*, *Triphyophyllum*, *Nepenthes* and a number of related noncarnivorous genera (Fig. 1; Albert *et al.*, 1992; Williams *et al.*, 1994; Lledo *et al.*, 1998; Meimberg *et al.*, 1999). Sepals of *Plumbago auriculata* Lam. (=*P. capensis* Thunb.) and *Plumbago indica* L. bear large mucilage secreting trichomes, which resemble those of *Drosophyllum* (Rashmilevitz and Joel, 1976). If *Plumbago* is carnivorous it would extend the carnivorous syndrome to groups basal to the clade of caryophyllacious carnivorous plants that has been revealed by cladistic analysis of molecular characters (Fig. 1). The objective of this study is to observe if *Plumbago* trichomes are capable of capturing insects and if they are capable of being stimulated to produce digestive enzymes.

Materials and Methods

Plant materials consisted of *Plumbago auriculata* (=*capensis*) and *Plumbago indica* were from Logee's Greenhouses, Danielson, CT, U.S.A. grown in a greenhouse in a 14 hr daylength.

The substrate film method (Hartmeyer, 1997) as modified by Carroll and Darnowski (2001) at Washington College for use on trigger plants was adapted for use on *Plumbago* flowers. On the first day of the three-day procedure the sepal was stimulated by brushing on the solution that was being tested. Each side of the calex was stroked twice with a freshly dipped brush. After stimulation with NaCl, NH_4Cl, and KCl and yeast extract solutions, the plants were left in the greenhouse for 24 hr. On the second day the flowers were clipped at the base of the pedicle and laid on a piece of Kodak 400 film which had previously been exposed to fluorescent light for a brief time then professionally developed. Just previous to the experiment the segments of film were coated with a solution of KH_2PO_4 buffer to prevent acid hydrolysis of the gelatin emulation. The flower and sepal were applied to the film in a Petri dish, containing a moist paper towel and the Petri dish was sealed with Parafilm and placed under fluorescent lights for 24 hr. The third day the film was removed form the dish

and dipped in tap water to remove any digested gelatin after which the film was allowed to dry. Negative controls involved placing drops of KCl, NaCl, NH_4Cl solutions and double distilled water on the gelatin emulsion film. Positive controls used with *Drosera capensis*, known to secrete proteolytic enzymes. The quantity of gelatin digested from the film surface indicated the quantity of proteolytic enzymes secreted.

Paraffin and plastic embedded sections of *Plumbago auriculata* were produced by cutting the sepals in half longitudinally with a razor blade and fixing them. Paraffin sections were made using Forssmann's procedure (Forssmann *et al.*, 1977). Plastic embedded sections were made by fixing using Kelley's OTO procedure (Kelley *et al.*, 1973), embedding in JB4 Historesin blocks and sectioning with an LKB microtome. The sections were heat fixed then stained. Fresh tissue was frozen and sectioned in a cryostat. Sections were stained at two times that recommended for animal tissue with PAS-Shift's reagent, alcian blue, safranin, crystal violet, toluadine blue, Delafield's hematoxylin and orange G.

Results

Capture of insects occurs on the trichomes of both *Plumbago auriculata* and *Plumbago indica*. We have observed and photographed captured insects both in the field in Florida and in the greenhouse (Fig 2). Observations are limited and it is not certain how many insects are captured but it is clear that insects are captured by the trichomes on *Plumbago* sepals. Insects observed on the trichomes were: ants, spiders, gnats, and flies.

Protease secretion from trichomes on sepals of *Plumbago auriculata* and *Plumbago indica* occurs in response to stimulation by several salts. However, yeast extract, which is capable of stimulating secretion of proteases in both *Drosera* and *Stylidium* trichomes (Carroll and Darnowski, 2001), fails to stimulate secretion in *Plumbago auriculata* and *Plumbago indica* (Fig 4). It has long been known that Na^+ and NH_4^+ stimulate secretion in *Drosera* tentacles (Darwin, 1893) so these ions were tested on the two species of *Plumbago* and *Drosera capensis* as well. Both NaCl and NH_4Cl stimulate secretion of protease at 5.0 mM concentrations in *Plumbago auriculata* (Fig. 3). KCl has less of an effect (Fig. 3) at the same concentrations. The 5 mM NaCl and NH_4Cl both stimulate secretion of protease by *Plumbago indica* as well. The secretion of protease increased as the concentration of the stimulus was increased as was evidenced by the more complete digestion in response to each of the stimuli (Fig. 3). This was true for *Plumbago auriculata*, *Plumbago indica*, and also *Drosera capensis*.

Stimulation by captured insects also resulted in proteases being secreted but it is unclear whether the protease comes from the insect or from plant secretions.

Mechanical stimuli seemed to have no effect. Preliminary experiments with a vibrating brush showed that mechanical stimulation alone did not increase the secretion of proteases. It is possible that mechanical stimulation might enhance the response to chemical stimulation as it has been shown to do in *Drosera capensis* (Lloyd, 1942).

Anatomy of the trichome resembles *Drosophyllum* stalked glands but is not precisely the same (Figs. 4, 5). A thicker layer of gland cells forms a cap above an endodermis similar to *Drosophyllum*. The stalk has an epidermis, a outer layer cortical parenchyma 3-5 cells thick and an inner core of narrow elongated parenchyma cells 7-12 layers thick (Figs. 4,5). The stalk is far thicker than that of *Drosophyllum* but lacks its pronounced vascular-ization. In at least some trichomes there appears to be a single vessel element entering the stalk but further investigation of the consistency of this feature is needed. The secretion of unstimulated trichomes stains positive with Sudan IV and negative with PAS indicating a lipid rather than a muscilaginous secretion like that found in *Drosophyllum*.

Discussion

Both *Plumbago auriculata* and *Plumbago indica* are capable of capturing insects and

secreting proteases in response to stimuli that would be expected to be present on the surface of insects and in decomposing insects. The absorption and assimilation of the material from captured prey has not been demonstrated and will be the subject of future study.

It is probable that *Plumbago*, like *Stylidium*, is carnivorous during flowering. This is a period when plants have a maximum demand for minerals since nutrients are being drained from the parent plant by seed production. It is likely that this type of carnivorous adaptation is more wide spread. Lloyd (Suda *et al.*, 2002) divided secretion on the glands of plants that catch small insects into three groups: oily (often aromatic), resinous, and mucilaginous. He states that carnivorous plants with adhesive traps all have mucilaginous secretions. It stands to reason that digestive and absorptive processes should more readily occur in an aqueous solution. Rachmilevitz and Joel (1976) reported that glands on *Plumbago* sepals have a resinous secretion. The secretion of unstimulated glands stains positive with Sudan IV and, therefore, must have a substantial hydrophobic constituent. Experiments testing the chemistry of stimulated glands are planned in the future.

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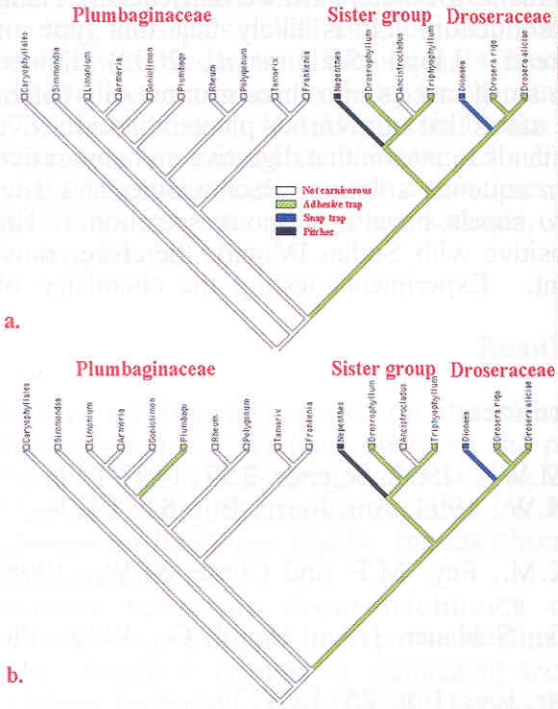

Fig 1. a) A phylogeny of Droseraceae, and related genera based on a cladistic analysis of the mat K gene (4). Carnivorous caryophyllids form a form a single clade Plumbago is related but not in the carnivorous clade

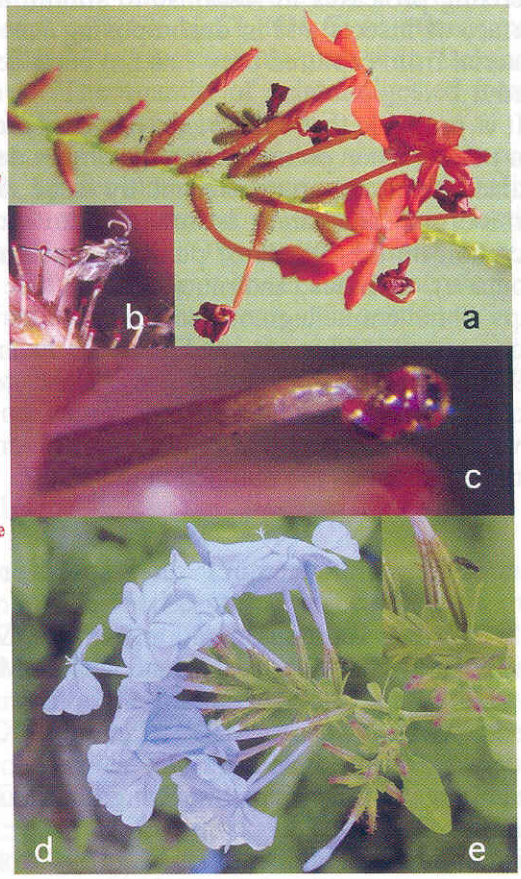

Fig. 2. a,b,c *Plumbago indica*; a) Inflor-escence, b) A captured fungus gnat, c) Trichome (150X). d,e *P. auriculata*; d) Inflorescence e) a captured spider.

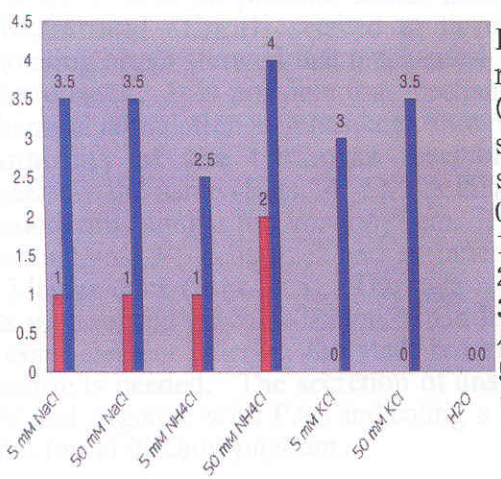

Fig. 3. A comparison of the relative responses of *Plumbago auriculata* (Blue) and *Plumbago indica* (Red) to chemical stimuli. Numbers show median gelatin substrate digestion after stimulation.
0 = no digestion
1 = pitting numbering < 20
2 = extensive pitting > 20 but no clear spots
3 = small clear holes ≤ 1 mm in diameter
4 = medium clear holes > 1 mm & < 5 mm in diameter
5 = large clear holes > 5 mm in diameter
* The values on the vertical scale are the median of six samples.

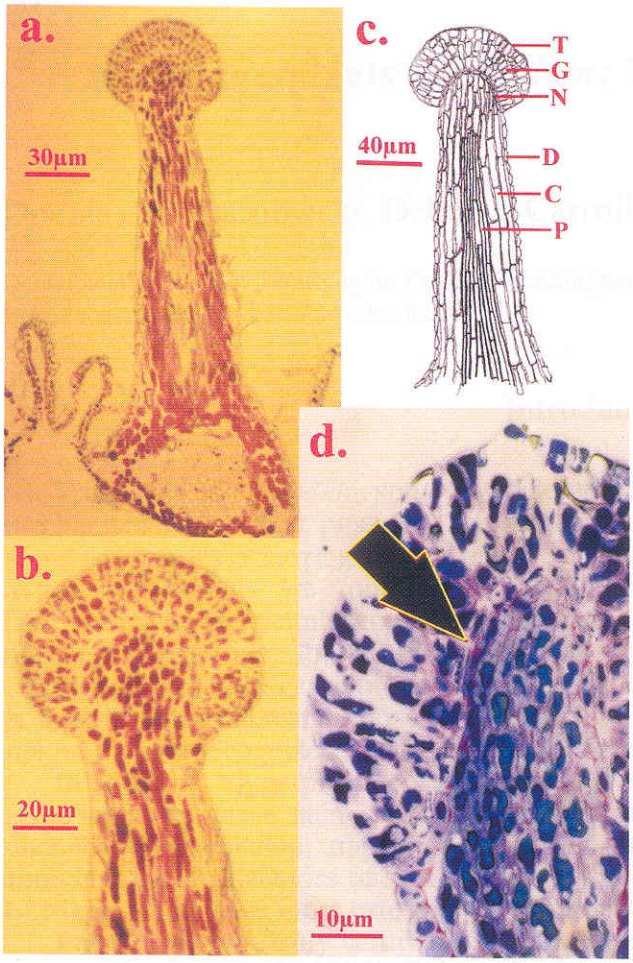

Fig. 4. *Plumbago auriculata*
a) (PAS) , b) l-section of trichome; c) Structures of trichome: G-gland cells, D-epidermis, T-cuticle, C-cortical parenchyma, P-elongated parenchyma, N-endodermis (also in "d" arrow points to casparian strip); d) (toluidine blue)

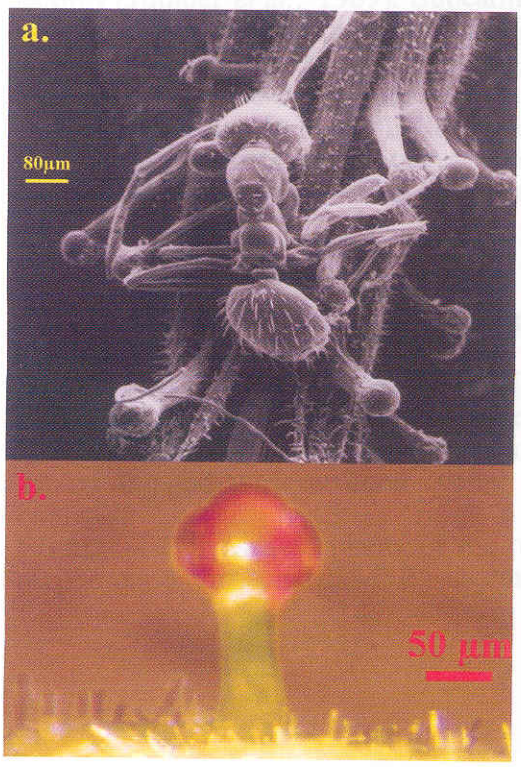

Fig. 5. *Plumbago auriculata*:
a) (SEM) of an ant captured on a sepal.
b) A live trichome illustrating the secretion droplet. The shape and angle of the stalked glands can be seen, as well as the hairs on the sepal.

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Are triggerplants (*Stylidium*; Stylidiaceae) Carnivorous?

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Introduction

Triggerplants (*Stylidium* spp.; Stylidiaceae) take their name from the explosive pollination mechanism which they possess. Triggerplants are nearly endemic to Australia, with only about ten species occurring in Sri Lanka, Southeast Asia, and New Guinea (Ericson, 1981). When triggerplants are found in the wild, they usually grow very near well-known carnivorous and subcarnivorous plants like *Drosera*, *Utricularia*, and *Byblis*. Since poor soils are often cited as a reason for the presence and success of carnivorous plants, theassociation of triggerplants with carnivorous plants may indicate that triggerplants are carnivorous. This is a particularly interesting possibility given the recently identified abilities of several plants not previously recognized as carnivorous (Spomer, 1999).

In addition, triggerplants possess glandular hairs which secrete a sticky substance. The secretion from these hairs, which resembles mucilage but which requires further precise chemical determination, traps insects on cultivated plants. These hairs are almost always found on the abaxial sides of flower parts as well as on pedicels and peduncles. Some species of triggerplants have more extensive areas of hairs (Darnowski, 2002)

For a plant to be truly carnivorous, it must trap and digest prey and absorb nutrients from its prey (Juniper *et al.*, 1989). Subcarnivorous plants like *Byblis* trap and absorb but rely on assistance from other organisms for digestion of their prey. The ongoing project described here deals with all three parts--trapping, digestion, and absorption. Trapping by triggerplants has been observed in the greenhouse in the USA, in the field in Australia, and in herbarium specimens in Australia and in the USA, while digestion has been explored using film tests. Absorption is being studied using a novel fluorescent compound since radioisotopes cannot be used at the author's institution.

Materials and Methods

Herbarium Multiple specimens of various triggerplants were observed in the L. H. Bailey Hortorium's herbarium at Cornell University, Ithaca, New York, U.S.A., and at the Herbarium of the Royal Botanical Gardens, Sydney, Australia. The number and type of insects trapped was recorded, using known carnivorous and non-carnivorous plants as controls.

Plants For assays, plants of the tropical *S. fimbriatum* were cultivated *in vitro* under aseptic conditions on 1/5 MSB5 medium pH 5.8 before autoclaving supplemented with 3%

(w/v) sucrose and 0.8% Bactoagar. Some plants were cultivated in terraria outside aseptic culture since more material could be produced in this way, and plants of the Tree Triggerplant (*S. laricifolium*) were cultivated in a cool, frost-free greenhouse in a mixture of sand, long fiber sphagnum moss, and sphagnum peat.

Film assay Photographic emulsions are made of silver halide crystals embedded in an emulsion of protein. When exposed, developed film, which is black, is digested by proteases from any source, the protein is removed and a clear hole appears (Heslop-Harrison and Knox 1970). The multiple, colored layers of emulsion in color film provide greater sensitivity since partial digestion can be seen as colored areas (Fratello, 1968).

Plants usually need to be induced to secrete digestive enzymes. This induction can be accomplished simply using a solution of 0.02 g per l of yeast extract in water, determined in preliminary experiments based on Hartmeyer (1997). This solution was applied 2 d before testing was to occur. Then excised induced organs were placed on pieces of exposed developed color negative film (Kodak Gold 400 ASA; Kodak, Rochester, New York, USA) for 2 d in sealed, humidified dishes. At the end of this time plant material was carefully removed, to avoid tearing the moist, tender emulsion, gently rinsed, and air dried. Digested pieces of film were scanned using an Olympus 1240U scanner, and levels, brightness, and contrast were adjusted using Adobe Photoshop 5.0LE.

Uptake synthesis Because it is not possible to use radioisotopes to follow uptake of nutrients at the authors' institution, the synthesis of fluorescein-glutamine, for use in following uptake of nutrients by *S. fimbriatum*, has been pursued. Fluorescein-isothiocyanate (FITC) was reacted with glutamine in a $NaHCO_3$ buffer according to Strottman *et al.* (1983) and quenched with milk powder (casein). This yields two products, fluorescein-glutamine and fluorecein-casein which can be used for uptake studies using fluorescence microscopy. The former compound was selected for these studies due to it's higher fluorescence yield--not all amino acids in casein can react with FITC while all of the glutamine can react at both primary amino groups. After electrophoretic separation by SDS-PAGE, fluorescein-glutamine was isolated by cutting the gel, macerating, and extracting with water using a syringe and filter.

Results

Herbarium and field When triggerplants and other plants were observed in the field, the following pattern was observed:

Table 1. Trapping of insects

Plant	Trapping
Non-carnivorous	None
Australian annual sundews	Numerous
Australian tuberous and pygmy sundews	Some *
Australian triggerplants	Some/None--varies by species; *S. musicola* and *S. graminifolium* had some of highest numbers trapped, numbers similar to *

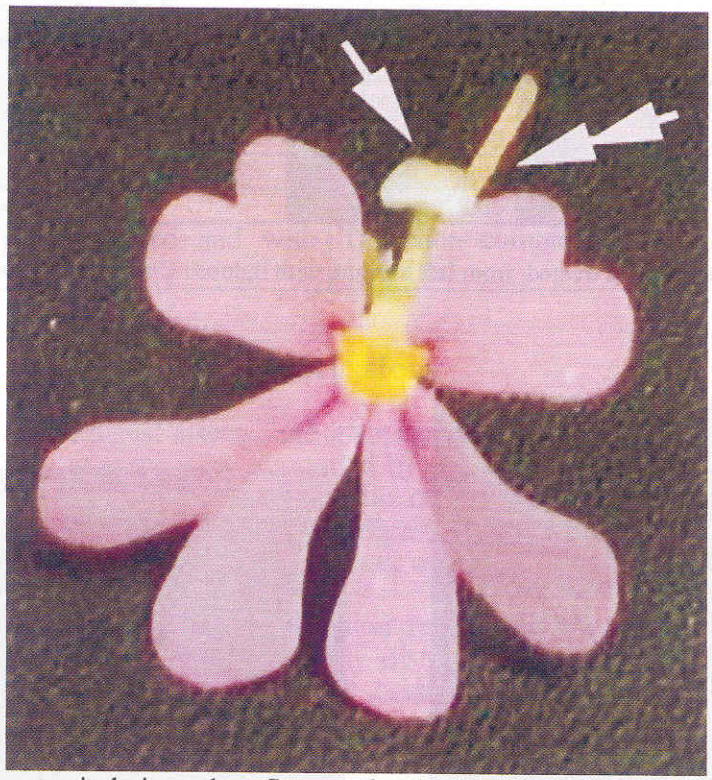


Figure 1 A flower from a tropical triggerplant, *S. musicola*. The single arrowhead indicates the trigger involved in the explosive pollination mechanism, and the double arrowhead indicates one region where glandular hairs are found. The flower is about 1 cm wide.

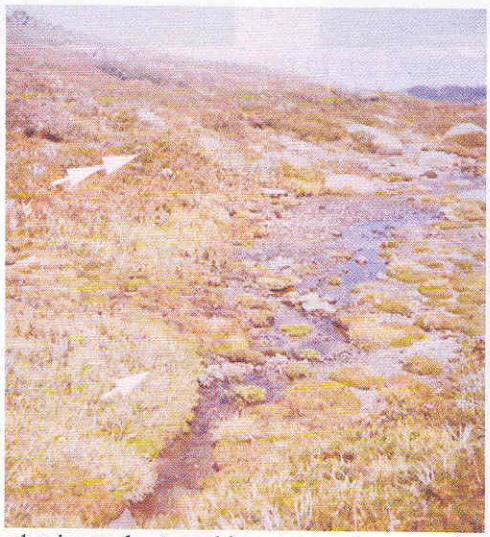
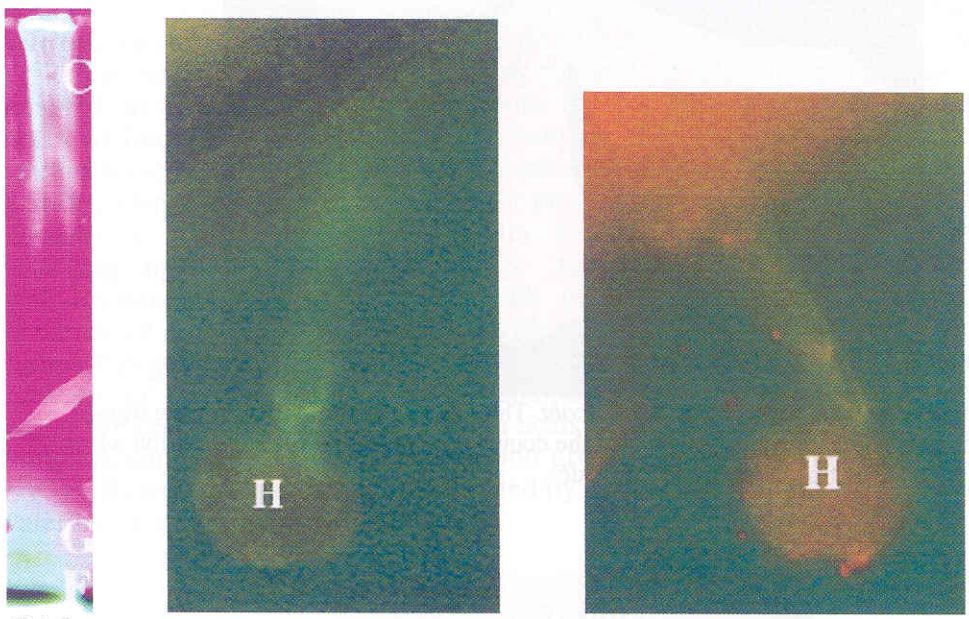


Figure 2 A location containing both triggerplants and known carnivorous plants. This stream in an alpine area of New South Wales contains both *Drosera arcturi* closer to the water (single arrowhead) and the Grass Triggerplant (*S. graminifolium*; double arrowhead; from Darnowski 2002) in slightly drier areas.

Film tests

Fig 3 Photographic emulsions treated, from left to right, with induced Citrus leaf, induced sundew leaf (about 3x life size).



Uptake

Figure 4 One lane of a gel, showing greenish fluorescence of casein-fluorescein (C), glutamine-fluorescein (just below G), and unreacted fluorescein (F).

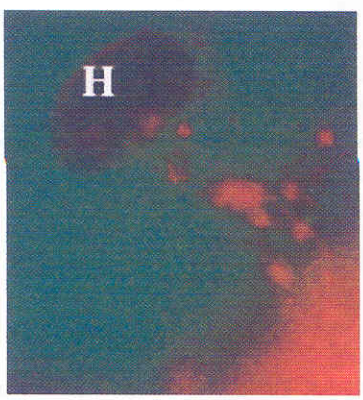


Figure 5 Uptake of fluorescein-glutamine by triggerplants. From left to right, negative control, S. fimbriatum induced with water only, S. fimbriatum induced with yeast extract. Greenish fluorescence is luorescein-glutamine. H=head of glandular hair

Table 2. Digestion of photographic emulsion by various materials

Plant Material	Result
Any material without induction	No digestion or very slight (sundew)
Citrus leaf with induction	No digestion
Sundew with induction	Strong digestion
Triggerplant with induction	Strong digestion
Sundew or triggerplant induced and with inhibitors	Digestion completely or nearly completely inhibited

Discussion

Triggerplants trap and digest insects, and it appears that this digestion is performed by the plants themselves. This is particularly supported by the digestion of photographic emulsion by plants grown in aseptic culture, in which no other organisms are present to assist in digestion, and by the specific inhibition of this digestion by a mixture of protease inhibitors. Also supporting this argument are the co-ocurrence of triggerplants and accepted carnivorous plants in the wild in Australia.

In future, a number of lines of investigation remain, including investigation of many species of *Stylidium* to determine how widely distributed carnivory is in this genus and whether it is found in *Stylidium*'s sister genus *Levenhookia* and in any other genera in the Stylidiaceae, which are also found growing in environments with accepted carnivorous plants.

The enzymology of triggerplants will be explored further using individual protease inhibitors in a combinatorial fashion to determine which specific proteolytic activities are secreted by triggerplants--for example metalloproteases or tyrpsin-like proteases. Further work will also be performed on uptake of fluorescent compounds to establish firmly this aspect of the carnivory of triggerplants.

Acknowledgements

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A half-century old of the Insectivorous Plant Society, Japan

Yoshiyuki Sodekawa[1] and Chiaki Shibata[2]

[1]Insectivorous Plant Society, [2]Department of Biology, Nippon Dental University

Outline of our Society

Organization name:	The Insectivorous Plant Society (IPS),Japan
Address:	Department of Biology, Nippon Dental University Fujimi, Chiyoda-ku, Tokyo 102-8159
E-mail	http://www2.odn.ne.jp/~chr79360/
Establishment:	November, 1949
Chairman:	Sadashi Komiya
Vice Chairman:	Momozo Tanaka and Yoshiyuki Sodekawa
Secretary:	Chiaki Shibata
Committee members:	15 persons
Members:	530 persons
Journal:	quarterly, the first Journal was published in January 1950 and most current issue of No.180 was published in April 2002.
Meetings:	4~6 times a year and several excursion to the CP sites in Japan and abroad

Detailed Introduction of IPS

[Establishment]

IPS was established in November 1949. It is assumed to be the oldest CP society in the world. IPS was named after Charles Darwin's book "Insectivorous Plants." We held a party celebrating our 50th anniversary of establishment in November 1999 in Tokyo. It gave us a good opportunity to enjoy reunion of the original members who now live in every corners of Japan.

[Members]

The members of IPS amount to approximately 530 from all over Japan. (We have a few overseas members so far.) They consist of CP lovers and enthusiasts as well as nature conservatists. Our youngest member is about ten years old wheras the oldest one is over 90 years old. The profession varies: students, businessmen, scholars, accounting professionals, managers of private owned companies, and so on. There is no dominant profession among the members.

[Activities]

Meetings: We have regular meeting five times a year. The venue is the NipponDental University at Iidabashi in Tokyo. The meetings are usually held in January, April, June, October, and November. The proceedings of the meeting includes: I. AttractionÅ Often a report of visiting CP site abroad by members. Video and slides are shown in the presentation. II. Exhibition of CP plantsÅ We bring our beloved cultivating plants. Owners explain how they love their plants as well as how to cultivate their plants.

I. Exchange of PlantsÅ We bring at least one pot of plants with each other that we

want others to keep. The master of ceremony performs a BINGO games. The winners choose a plant they want. He or She has to propagate it and bring it back to the Ågexchange marketÅhsomeday.

II. Selling and Buying plantsÅ We sell and buy plants and seeds that are in excess at his or her house at a reasonable price. This is to enhance circulation of plants that we can seldom get at a market.

All these events are executed in four hours in a day.

Observation and conservation of CP sites in Japan: We execute several excursions to the CP sites in Japan for benchmark observation. They include Mt.Koshin for *Pinguicula ramose* in June, Kikukawa Town in Shizuoka Pref. for *Drosera peltata*, Icchoda bog in Aichi Pref. for *D. indica* and *Utricularia minutissima*, Hanyu(Please pronounce Å gHa-newÅ h City in Saitama Pref. for *Aldrovanda vesiculosa* ,and so on.

Dr.Komiya and Ms.Shibata visit the *P. ramose* and *A. vesiculosa* site several times a year and have accumulated scientific data for conserving those plants. These excursions are participated on voluntarily base.

Exhibition: Every summer we hold an exhibition of CPs at Isetan department store in Shinjuku,Tokyo. The exhibition is held in the late July when school boys and girls enjoy their summer vacation and have time to visit. We also prepare plants for sale. This is a good opportunity for expanding our members and letting the ordinary people to discover and understand the reality of CPs.

Journal: Our journal is issued quarterly. The topics include tips of cultivation, reports of visiting CP sites in Japan and abroad, benchmark observation of valuable CP sites in Japan, introduction of newly published CP books and so on. It includes some color photos inside pages as well as the front and the back pages. The journal is in Japanese only.

[Visiting CP sites abroad]

In recent years we regularly visit CP site abroad. Ms. Chiaki Shibata is leading this project. She visited Guyana Highland several times (1993~2000). In 1995 and 1997 we visited Sakhalin of Russia in search of *Pinguicula variegata* . In 1998 and 2000 we visited Brazil to meet beautiful *Drosera*, *Utricularia*, *Genlisea*, and *Paepalanthus*. In 1996, 1999, 2000 and 2001 we visited Australia to meet various types of *Drosera*, pygmy, tuberous, and tropical, as well as *Byblis*. We also visited *Cephalotus* site to learn how the plants are growing in the natural habitat. We also succeeded in finding some sites of red *Aldrovanda*. In 2000 and 2001 we visited the U.S.to meet *Darlingtonia*, *Sarracenia* and *Dionaea*.

[Publications]

For people who get CPs by any chance, we have published several guide books to provide information on how to identify and cultivate CPs. These books are co-authored and compiled among our members. Unfortunately, those books are out of print now and a new book is being prepared.

Photo Album, *Insectivorous Plants*, Shimizu 1996
Garden Series, *Carnivorous Plants* 1979
Insectivorous Plants: In search of their wonder and secrets , Komiya 1994
Flower Album Series, *Carnivorous Plants* 1996

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Some carnivorous plants remained less-known in Japan

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Planthunter, 1-15-35, Houjyou, Daitou City, Osaka Prefecture 574-0011, Japan

Abstract. Some carnivorous plants collected by the author and rather be uncommon to Japan up to date are tabulated and shown by photography.

The carnivorous plants uncommon in the Japanese growers are listed as follows: South African CPs: *Drosera hilaris, D. ramentacea, D. glabripes, D. curviscapa, D. esterhynsena, D. pauciflora, D. zeyheri, D. alba, D.* sp. Floating; *Roridula gorgonias, R. dentata.* Zambian CPs: *Drosera affinis, D. elongata, D. biquaertii*; Venezuelan CPs: *Pinguicula elongata.*
The carnivorous plants uncommon and the author discovered: South African CPs: two unidentified species of *Drosera* collected in Gifberg; one unidentified species of *Drosera* each from Hixriver, Simonstown, Mont Rochere, Berbaton, Riversdale. Zambian CPs: two unidentified species of *Genlisea.* Australian CPs: a species of *Drosera* in Table Land. Local races uncommon: *Drosera cistiflora, D. pauciflora, D. hilaris, D. ramentacea, D. glabripes, D. alba*, and so on.

REGISTERED MEMBERS

FOREIGN MEMBERS

Name	COUNTRY
ADAM, Jumaat	Malaysia
ADAMEC, Lubomir	Czech Republic
ANFRAIX, Romuald	French Republic
CANTLEY, Robert	Democratic Socialist Republic of Sri Lanka
CAROW, Thomas	Federal Republic of Germany
CLARKE, Charles M.	People's Republic of China
CLAYTON, Colin H.	Australia
CLAYTON, Tina	Australia
DARNOWSKI, Douglas W.	The United States of America
FAGERBERG, Wayne R.	The United States of America
FRAZIER, Christopher K.	The United States of America
HARBARTH, Peter	Federal Republic of Germany
HARTMEYER, Irmgard	Federal Republic of Germany
HARTMEYER, Siegfried	Federal Republic of Germany
JOEL, Daniel M.	State of Israel
JOEL, Alma	State of Israel
JOEL, Rachel	State of Israel
KAMINSKI, Ryszard	Republic of Poland
KIM, Shinje	Republic of Korea
KOSIBA, Piotr	Republic of Poland
LANE, David M.	The United States of America
LEE, Ch'ien C.	Malaysia
LEE, Soon-Bong	Republic of Korea
LEGENDRE, Laurent L.	Australia
LEGENDRE, P.	Australia
LUM, Greg C.	The United States of America
MURUGAN,C	Republic of India
MUTHURAMALINGAM, K. J.	Republic of India
OGG, Randy	The United States of America
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