

PREY CAPTURE PATTERNS IN *NEPENTHES* SPECIES AND NATURAL HYBRIDS –
ARE THE PITCHERS OF HYBRIDS AS EFFECTIVE AT TRAPPING PREY
AS THOSE OF THEIR PARENTS?

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Introduction

The carnivorous pitcher plant genus *Nepenthes* (Nepenthaceae) is thought to comprise more than 120 species, with a geographical range that extends from Madagascar and the Seychelles in the west, through Southeast Asia to New Caledonia in the east (Cheek & Jebb 2001; Chin *et al.* 2014). There are three foci of diversity – Borneo, Sumatra, and the Philippines – which account for more than 75% of all known species (Moran *et al.* 2013). The pitchers of *Nepenthes* have three main components – the pitcher cup, the peristome (a collar-like band of lignified tissue that lines the pitcher mouth), and the lid (Fig. 1A-G). In most species, the lid is broad and flat and overhangs the mouth (Fig. 1B-D), but in some specialized species it is small and oriented away from the mouth (Fig. 1A,E). The inner walls of the pitcher cup may be divided into two discrete zones – a lower “digestive” zone in which the pitcher walls lack a waxy cuticle and are lined with digestive glands; and an upper “conductive” zone, which lacks digestive glands but is covered by a complex array of wax crystals (Juniper *et al.* 1989; Bonhomme *et al.* 2011). Insects that make their way onto the conductive surface often lose their footing and fall into the digestive zone, which contains a viscoelastic fluid that facilitates the retention and drowning of prey.

Ever since *Nepenthes* were first encountered by Europeans, scientists have attempted to determine how the various components of the trap operate (Lloyd 1942; Phillipps & Lamb 1996). However, it was not until relatively recently that the structure and function of the peristome were properly elucidated, a discovery that has had a profound influence on subsequent research (Bohn & Federle 2003). It has been demonstrated that the peristome is a highly effective trapping surface when it is moist, but not when it is dry, due to its anisotropic, wettable surface microstructure (Bauer *et al.* 2008). By contrast, the effectiveness of the waxy zone is independent of moisture levels (Bauer *et al.* 2012a; Moran *et al.* 2013). An enlarged peristome is thought to have lower construction costs compared to a well-developed waxy zone (Poorter & De Jong 1997; Riedel 2007), so in perhumid environments (i.e., those that experience high levels of rainfall throughout the year and lack distinct dry seasons), traps with reduced waxy zones and expanded peristomes are likely to be the most efficient at trapping prey (Bauer *et al.* 2012a; Moran *et al.* 2013). However, in seasonal environments, a well-developed waxy zone and narrow peristome will be effective even if the weather is relatively dry for short periods. For this reason, the species with the largest, most spectacular pitchers are effectively confined to equatorial habitats in Southeast Asia that experience a perhumid climate, whereas the narrow peristome/extensive waxy zone trap format is found throughout the geographical range of the genus (Moran *et al.* 2013).

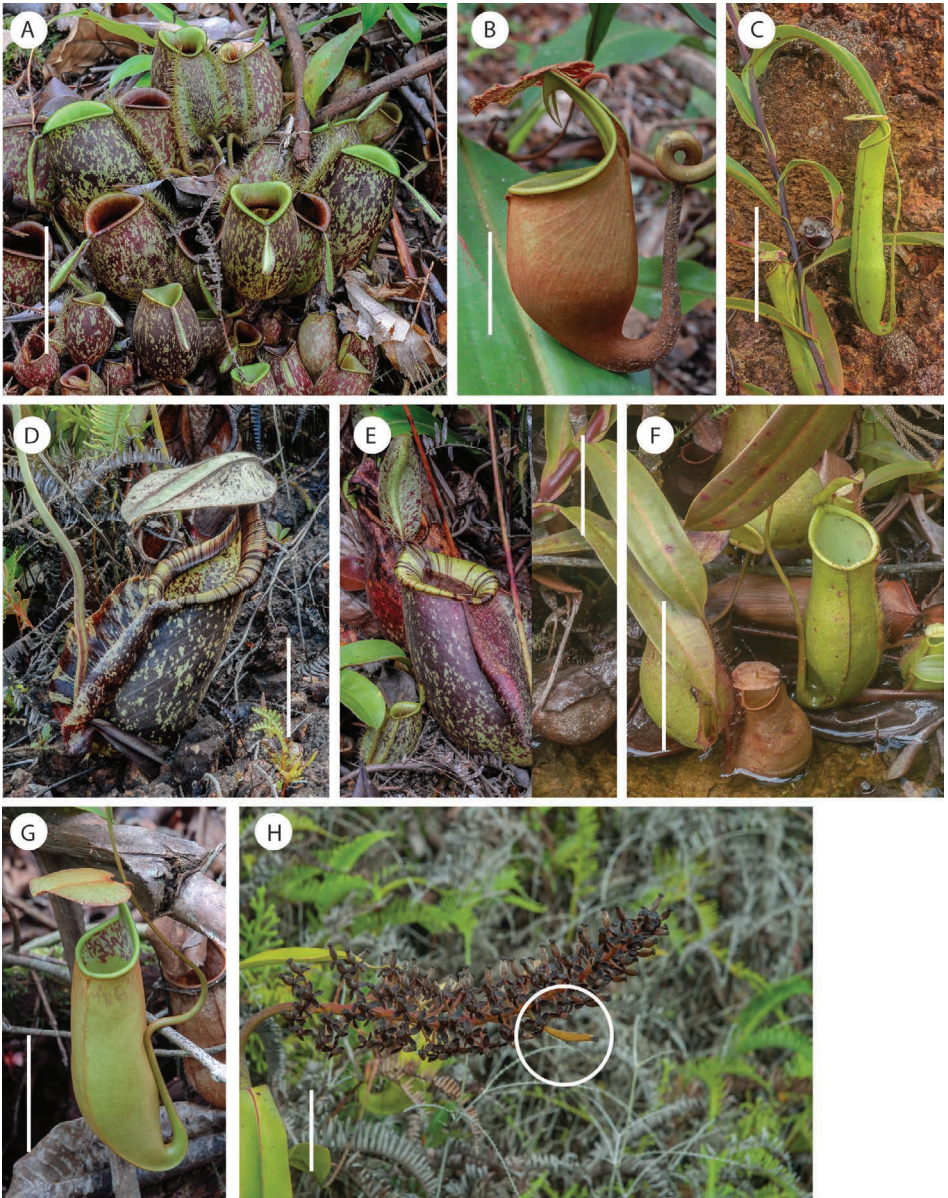


Figure 1: Representative images of the seven *Nepenthes* taxa included in this study. A. Terrestrial rosette pitchers of *Nepenthes ampullaria*, Mersing. B. Aerial pitcher of *Nepenthes bicalcarata*, Serian. C. Aerial pitcher of *Nepenthes gracilis*, Mersing. D. Terrestrial pitcher of *Nepenthes rafflesiana*, Mersing. E. Terrestrial pitcher of *Nepenthes* \times *hookeriana*, Mersing. F. Intermediate pitcher of *Nepenthes* \times *trichocarpa*, Matang. G. Aerial pitcher of *Nepenthes* \times *cantleyi*, Serian. H. Female inflorescence of *N. ampullaria* \times *N. gracilis*, Matang, Sarawak. Note the single fruit (circled). Scale bar in all images = 5 cm.

Open, moist sunny sites with poor soils are thought to provide optimal conditions for the carnivorous syndrome in plants (Givnish *et al.* 1984). Several common *Nepenthes* species, such as *N. gracilis*, *N. rafflesiana*, and *N. mirabilis*, frequently colonise sandy, nutrient-deficient substrates where the vegetation has been heavily disturbed, and, as these are among the commonest species in the Sunda region, this has led to a general impression among workers that the genus as a whole tends to prefer disturbed habitats (Phillipps & Lamb 1996; Clarke 1997). In fact, apart from a small number of species that are well-adapted to highly disturbed habitats, the majority of species grow in undisturbed vegetation that occurs on nutrient-deficient soils, such as tropical heath, peat swamp and upper montane forests, and/or grow as epiphytes in intact forest canopies (Clarke 2001; Chin *et al.* 2014). The vegetation in disturbed sites is often similar in structure to these formations (i.e., having a low, open canopy, patches of bare ground and an abundance of small shrubs), but as the majority of biologists who work with *Nepenthes* have tended to focus their efforts on a few widespread, common *Nepenthes* species that grow in disturbed sites, a skewed perception of what represents a “typical habitat for *Nepenthes*” has arisen.

Hybrids and hybridization in *Nepenthes*

This misconception has given rise to another: that *Nepenthes* frequently produce natural hybrids in the wild. *Nepenthes* are dioecious and produce fertile hybrids, and many putative natural hybrids have been reported (Clarke 1997, 2001). In suitable habitats in Borneo, Sumatra and the southern Philippines, it is not unusual to encounter two or more *Nepenthes* species growing in mixed populations at the same locality. If the vegetation at such sites has been recently disturbed (as is the case in most field sites used in previous research), natural hybrids may be common, but in undisturbed habitats, natural hybrids are actually very rare. The most likely explanation for this is that the majority of co-occurring *Nepenthes* species in undisturbed sites appear to be reproductively isolated due to staggered flowering times (C. Clarke, pers. observ.). However, disturbances to the plants’ habitats can disrupt the normal flowering seasons, resulting in overlap between species that are normally temporally isolated with regards to their phenology. When this happens, cross-pollination may occur, giving rise to hybrid progeny. There is little documented evidence to support these patterns at present – again; this is because few biologists have conducted detailed surveys of *Nepenthes* phenology in undisturbed habitats. One (qualitative) example involves mixed, co-occurring populations of *N. veitchii*, *N. hurrelliana* and *N. chaniana* on Mt. Batu Buli in northern Sarawak. In 1988, one of us (CC) visited this mountain which, at the time, supported pristine montane forest. The three species of *Nepenthes* mentioned above were common in this forest, but only one or two putative hybrids were found among a multi-species population that numbered in the thousands of plants. On a subsequent visit to the same area in 2007, a few years after the site had been disturbed by the construction of a logging road (and subsequent removal of many of the canopy trees in the area), the overall number of plants at the site was much reduced, but putative hybrids involving all three species were common, possibly accounting for as much as 10% of the *Nepenthes* plants that grew along the old logging road. At similar disturbed sites in the lowlands of northwestern Borneo, natural hybrids were observed by CC to be common in the late 1980’s, but once the disturbances in this area ceased and the forest recovered (c. 2012) most of the hybrids had died out, whereas the parent species remained.

Thus, based on the meager, qualitative evidence available, it seems that most *Nepenthes* species that grow in undisturbed habitats rarely produce natural hybrids, but when significant habitat disturbances occur, hybridization is more common. Furthermore, in the absence of ongoing habitat disturbances, hybrid plants appear to be unlikely to persist and form independent, stabilized populations of their own. Given that the few phylogenetic studies of *Nepenthes* based on nucleic acids (e.g.,

Meimberg & Heubl 2006; J. Mullins, unpublished) propose that introgression is widespread in the genus, it seems incongruous that hybrid plants appear to be “unsuccessful” at persisting in the wild.

There are two primary mechanisms by which natural hybrids can confer evolutionary advantage, either to the genus as a whole, and/or to one or both of their parent species. The first is that the hybrid plants themselves are more competitive (or have greater evolutionary fitness) than the parents, and are capable of reproducing effectively among themselves. Over time, the hybrid plants increase in number and become reproductively isolated from the parent species, eventually becoming established as a new species (Clarke 1997). Alternatively, the hybrid plants may fail to compete effectively, and/or become independent of the parents, but they may cross back (this is called introgression) with one or more parent species, thereby increasing the size of the gene pool of the parent species and enhancing its ability to respond to changes in selective pressures that might arise, say, from processes such as climate change or habitat disturbance (Clarke 2001).

Prey capture in *Nepenthes* and the characteristics of *Nepenthes* hybrids

It is common for the pitchers of natural *Nepenthes* hybrids to appear “intermediate” between those of their parent species (Clarke 1997). For instance, if one species that produces narrow, tubular pitchers with well-developed waxy zones (e.g., *N. gracilis* (Fig. 1C)) is crossed with another that produces squat, ovoid ones that lack a waxy zone, but have a large, expanded peristome (e.g., *N. ampullaria* (Fig. 1A)), the pitchers of the offspring tend to be broader and more ovoid than those of *N. gracilis*, but taller and narrower than those of *N. ampullaria*, with a small waxy zone and a slightly expanded peristome (e.g., *N. ampullaria* × *N. gracilis* (= *N. × trichocarpa*), Fig. 1F). Clarke (1997) examined the metazoan invertebrate community from pitchers of *N. bicalcarata*, *N. gracilis* and the natural hybrid, *N. bicalcarata* × *N. gracilis* (= *N. × cantleyi*) (Fig. 1B, C, and G), and found that even the structure and composition of the animal community of the hybrid was intermediate between those of its parent species. Given that both *N. bicalcarata* and *N. gracilis* have been shown to have specialized trap function (Clarke & Kitching 1995; Bauer *et al.* 2012b), what are the implications of “being intermediate” for prey capture by hybrid *Nepenthes*?

Recent research into nutrient acquisition in *Nepenthes* has demonstrated that species with highly specialized pitcher structures tend to have highly specialized nutrient sequestration strategies. In other words, species that target a particular type of prey appear to produce pitchers with modifications that make them particularly attractive to that prey (Chin *et al.* 2010; Chin *et al.* 2014). One example is *N. albomarginata*, which deploys a band of dense white hairs beneath the peristome that attracts termites, which feed on the hairs and are trapped in large numbers by the pitchers (Moran *et al.* 2001; Merbach *et al.* 2002). Furthermore, *N. ampullaria* traps falling leaf litter in addition to insects by producing a “carpet” of pitchers at ground level (Fig. 1A), that have small, reflexed lids that do not cover the pitcher mouths. This allows falling detritus to enter the pitchers freely, and such inputs account for a significant proportion of foliar N in this species (Moran *et al.* 2003). More recently, the outsized, highly modified pitchers of *N. lowii*, *N. rajah* and *N. macrophylla* have been shown to attract mountain tree shrews (*Tupaia montana*), which defecate into the pitchers in return for a reward of nutritious nectar provided by glands on the pitchers’ lids (Clarke *et al.* 2009; Chin *et al.* 2010).

Chin *et al.* (2014) posed the question: if gross modifications to *Nepenthes* pitcher characteristics facilitate specialized nutrient sequestration strategies, what about species that lack these? Do species that lack obvious pitcher specializations behave as generalist predators, trapping whatever insects happen to encounter the pitchers, or could they also be specialists, targeting specific types of arthropods over others, using subtle specializations to trap structure that are not obvious to human observers? To investigate this question, they studied prey capture patterns in eight *Nepenthes*

species from three localities in Borneo. Of these, four species produce pitchers with unique characteristics and specialized function (*N. ampullaria*, *N. bicalcarata*, *N. lowii*, *N. macrophylla*), whereas the remainder (*N. gracilis*, *N. mirabilis*, *N. rafflesiana*, *N. tentaculata*) do not. Their findings demonstrated when prey was identified to the taxonomic level of Order, the species with specialized pitchers could be easily distinguished through simple quantitative analyses, but that differences in prey capture strategies among the “non-specialists” were subtle and not readily explained. In all lowland species examined to date, ants (Formicidae) were the numerically dominant prey taxon, and it was only when the ants were identified to the level of genus and/or species that significant interspecific differences in prey capture strategies among these *Nepenthes* became evident.

Chin *et al.* (2014) concluded that it is possible that co-occurring *Nepenthes* species that trap mostly ants, using pitchers that lack gross morphological modifications, could target different combinations of ant taxa (presumably using subtle, or less obvious adaptations), thereby avoiding competition for prey. However, they also noted that the evidence they obtained to support this conclusion was not compelling and there may be alternative explanations for the patterns they found. However, if co-occurring *Nepenthes* do target different combinations of arthropod taxa, then comparison of their prey spectra to those of their natural hybrids could provide further insights into interspecific differences in prey capture patterns, as well as the effectiveness of hybrid pitchers in trapping prey. Such insights might also shed light on the ecological fitness of natural hybrids (or lack thereof).

Do hybrid *Nepenthes* trap prey as effectively as their parent species?

In species that have similar pitcher structure, such as *N. gracilis* and *N. mirabilis*, the fact that the pitchers of most natural hybrids are intermediate in appearance between those of their parents does not result in any obvious loss of pitcher function. However, in hybrids that involve one or more highly specialized parent species (such as *N. ampullaria* or *N. bicalcarata*), specialized pitcher characters may be lost, greatly reduced or rendered ineffective. Returning to the example of *N. bicalcarata* × *N. gracilis*; the distinctive thorns of *N. bicalcarata* pitchers are reduced to small bumps in the hybrid (Fig. 1G), so that any function they may serve in *N. bicalcarata* pitchers is not inherited. In such cases, do hybrids such as *N. bicalcarata* × *N. gracilis* inherit “the worst of both worlds” – losing the specializations of their parents, and lacking the ability to target any arthropod prey groups effectively? If so, it is possible that hybrids such as this would have difficulty obtaining the nutrients required to reach maturity and flower and set viable seed. And, if so, this could go some way to explaining their inability to persist in the wild.

At present, we can do little but postulate about the evolutionary and ecological fitness of *Nepenthes* hybrids, as no detailed ecological observations or experiments have been performed. In this study, we sought to establish a platform upon which further research projects might be based, by comparing prey capture patterns of three natural *Nepenthes* hybrids and their co-occurring parent species at three locations in Malaysia. We compared measures of several physical attributes of the pitchers (to find out whether hybrid pitchers are indeed intermediate in structure compared to those of their parents), and analyzed patterns in prey capture to address the hypothesis that hybrid *Nepenthes* plants are less effective at trapping prey than their specialized parent species.

Materials and Methods

Nepenthes taxa

Seven *Nepenthes* taxa, including four species and three natural hybrids, were included in the survey. The selection criterion for species and hybrids was straightforward – in order to compare

patterns of arthropod prey capture using quantitative analyses, it was necessary to select hybrid taxa that grew in mixed populations with both of their parent species at the same site, and which were sufficiently common to satisfy the most fundamental assumptions of the analytical methods used. These constraints preclude all but the most commonly occurring natural hybrids and within Malaysia, we were only able to identify three hybrids and localities that were suitable for this type of study. These were: *N. ampullaria* × *N. gracilis* (= *N. × trichocarpa*), *N. bicalcarata* × *N. gracilis* (= *N. × cantleyi*), and *N. ampullaria* × *N. rafflesiana* (= *N. × hookeriana*).

Study sites

The study was performed at three geographically isolated sites in Malaysia: one near the town of Mersing in Johor (on the Malay Peninsula) and the other two near the city of Kuching in Sarawak on the island of Borneo. The selection criteria for study sites were based on two factors: accessibility and suitability for running extended field experiments, and abundance of two species of *Nepenthes* and their putative natural hybrid growing together (and hence, exposed to the same pool of potential prey organisms) at the site. The first site (called “Serian” in this study), was located to the SE of Kuching, at an altitude of 37 m above sea level (asl). Since this study was performed in 2010, the vegetation at this site has been destroyed, but when our experiments were conducted, it supported fragments of several types of vegetation, ranging from open, bare sandy ground to intact peat swamp forest. Originally, the area supported a mosaic of peat swamp forest and tropical heath forest (*kerangas*), much of which has now been cleared. Five species of *Nepenthes* occurred at this site: *N. ampullaria*, *N. bicalcarata*, *N. gracilis*, *N. mirabilis* and *N. rafflesiana*. The species and hybrid that we surveyed at this site were *N. bicalcarata*, *N. gracilis* and *N. bicalcarata* × *N. gracilis*. Plants of *N. bicalcarata* were virtually confined to the intact peat swamp forest fragments, whereas *N. gracilis* grew at the margins of the intact forest and in open areas. Hybrids of these species grew both under the intact forest canopy and in open areas. The second site (referred to as “Matang”), was located on a roadside near the village of Matang, to the north of Kuching. Three species of *Nepenthes*, *N. ampullaria*, *N. gracilis* and *N. rafflesiana*, grow in mixed populations on open, exposed embankments on both sides of the road. The vegetation is sparse *adinandra belukar* (an anthropogenic shrubland formation that resembles degraded *kerangas* (Sim *et al.* 1992)), which is characterized by patches of bare ground, interspersed with patches of resam fern (*Dicranopteris linearis*) and small shrubs. This site was chosen because it also supports large numbers of the natural hybrid, *N. ampullaria* × *N. gracilis*.

The third site (called “Mersing”) was in the Malaysian state of Johor, near the town of Mersing, on the west coast of the Malay Peninsula. Like the Matang site in Sarawak, the plants grew in clearings by the sides of roads, in sparse *adinandra belukar* shrubland. Three species of *Nepenthes* (*N. ampullaria*, *N. gracilis*, and *Nepenthes rafflesiana*) occurred at this site, along with plants of two natural hybrids: *N. ampullaria* × *N. gracilis* and *N. ampullaria* × *N. rafflesiana*. Figure 1 presents photographs of all seven *Nepenthes* taxa included in the study.

Sampling methods

Pitchers were selected for study on the basis of their age and condition. Very old and very young pitchers, or those that displayed obvious signs of damage, may not be fully functional and hence were excluded. Maximum sample sizes were imposed by logistical constraints. We had sufficient human resources to study 10-30 pitchers of each species and hybrid at each site. The final numbers sampled were the number of pitchers that were still intact and operational at the end of the experiment (Table 1). All pitchers sampled were from separate plants. Where pronounced intra-specific pitcher dimorphism occurred, we attempted to sample equal numbers of both pitcher types, to ac-

Table 1. Summary statistics for prey capture by the seven <i>Nepenthes</i> taxa studied. Values are Means 1 ± S.D.						
<i>Nepenthes</i> species and location						
	Serian, Sarawak				Matang, Sarawak	
Prey inputs	<i>bicalcarata</i>	<i>gracilis</i>	× <i>cantleyi</i>	<i>ampullaria</i>	<i>gracilis</i>	× <i>trichocarpa</i>
Number of pitchers sampled	28	25	30	33	32	27
Mean number of individual prey items	38.607 ± 10.532	1.440 ± 0.664	5.867 ± 1.394	1.455 ± 0.348	3.156 ± 0.767	3.778 ± 1.743
Mean number of prey taxa	2.464 ± 0.311	0.600 ± 0.173	0.967 ± 0.153	0.667 ± 0.128	1.156 ± 0.156	0.852 ± 0.157
Arthropod Prey Taxa						
Coleoptera	0.321 ± 0.155	0.040 ± 0.040	0.067 ± 0.046	0.091 ± 0.091	0.156 ± 0.079	0.074 ± 0.051
Dictyoptera	0	0	0	0.030 ± 0.030	0	0
Hemiptera	0.107 ± 0.060	0	0.067 ± 0.046	0.030 ± 0.030	0	0.037 ± 0.037
Lepidoptera	0.107 ± 0.060	0	0	0	0.031 ± 0.031	0
Diptera	0.250 ± 0.083	0.160 ± 0.075	0.133 ± 0.078	0.152 ± 0.063	0.594 ± 0.190	0.259 ± 0.137
Orthoptera	0.071 ± 0.050	0	0.033 ± 0.033	0	0.031 ± 0.031	0
Formicidae	26.214 ± 7.621	1.040 ± 0.644	4.533 ± 1.245	1.152 ± 0.335	2.281 ± 0.760	3.407 ± 1.732
Arachnida	0.250 ± 0.098	0.040 ± 0.040	0	0	0.031 ± 0.031	0
Termitoidae	10.821 ± 3.939	0.120 ± 0.120	1.033 ± 0.641	0	0	0
Acarina	0.429 ± 0.181	0	0	0	0	0
Mersing, Johor						
Prey inputs	<i>ampullaria</i>	<i>gracilis</i>	<i>rafflesiana</i>	× <i>hookeriana</i>		× <i>trichocarpa</i>
Number of pitchers sampled	29	12	32	36		11
Mean number of individual prey items	2.172 ± 0.632	12.667 ± 10.773	8.000 ± 1.468	1.722 ± 0.427		1.364 ± 0.621
Mean number of prey taxa	0.759 ± 0.146	0.833 ± 0.167	1.688 ± 0.278	0.611 ± 0.121		0.818 ± 0.310
Arthropod Prey Taxa						
Coleoptera	0.069 ± 0.048	0	0.406 ± 0.173	0		0
Dictyoptera	0	0	0.156 ± 0.091	0		0
Hemiptera	0	0	0.031 ± 0.031	0		0
Lepidoptera	0	0	0.125 ± 0.087	0		0
Diptera	0	0.083 ± 0.083	0.250 ± 0.090	0		0.091 ± 0.095
Orthoptera	0	0	0	0		0.091 ± 0.095
Formicidae	1.828 ± 0.594	12.583 ± 10.778	5.656 ± 1.338	1.222 ± 0.359		1.091 ± 0.435
Arachnida	0.034 ± 0.034	0	0.156 ± 0.091	0		0.091 ± 0.095
Termitoidae	0.207 ± 0.144	0	1.125 ± 0.575	0.222 ± 0.127		0
Acarina	0.304 ± 0.304	0	0.063 ± 0.043	0.222 ± 0.155		0

count for potential effects of dimorphism. If more than 25 suitable pitchers of a given species could be found at a site, 25-35 of these were randomly selected for study. If less than 20 suitable pitchers were available, all of them were tagged and used.

Several physical characteristics of the pitchers were measured, including: The width and length of the pitcher orifice at their widest points, the width and length of the pitcher lid at their widest points, the width of the peristome at the side of the pitcher mouth, the pitcher height at the rear (measured from the base of the spur to the lowermost point of the pitcher), the pitcher height at the front (measured from the top of the peristome to the lowest point of the pitcher), the lengths of the wax zones at the front and rear of the pitcher, and the pitcher capacity. These characteristics may or may not have important roles in (targeted) prey capture strategies – they were chosen purely to assist in the description of overall pitcher structure and their potential roles in pitcher function were not investigated.

We used the method of Moran (1996) to survey arthropod prey capture in *Nepenthes* pitchers. This involves clearing the pitchers of their existing contents and “re-setting” them, so that they capture prey for a fixed, uniform period, thereby enabling direct comparison of capture rates among all pitchers. Pitchers were emptied of their contents and rinsed with distilled water. The contents were then passed through filter paper to remove all macroscopic detritus. The volume of the fluid was then measured to the nearest ml and returned to the pitcher, whereas the detritus was discarded. Pitchers were then left for 14 days to capture prey. This time interval was chosen as it allows the longest possible period for pitchers to trap prey without providing the invertebrate fauna sufficient time to re-colonize the pitchers and degrade the prey to the point where identification becomes difficult. At the conclusion of the experiment, the contents of the pitchers were poured into a 250 ml beaker and the inner surfaces of the pitchers were thoroughly rinsed with distilled water to remove all of the contents. The fluid was filtered once more, but this time the contents were retained and preserved in 70% ethanol for sorting and identification, whereas the filtrate was returned to the pitchers. Remains of captured prey were sorted and identified to the level of Order. Ants were identified to the subordinal rank (Formicidae) to distinguish them from other Hymenoptera, such as bees and wasps.

Data Analysis

All summary statistics are presented as means \pm 1 SD or SE (depending on the analysis). Descriptive statistics and principal components analyses were calculated and analysed using Minitab v. 16. All decisions about hypotheses were made against a critical value of $\alpha = 0.05$.

Quantitative analysis of prey capture patterns were conducted at the Ordinal level for all main arthropod taxa, except for ants (Formicidae) due to their importance as prey in lowland pitchers.

Prey capture patterns were depicted using a series of “star plots”, which display proportional abundances of prey taxa for each *Nepenthes* species, in which each taxon is represented by a “wedge” in a circular chart Ellison and Gotelli (2009). The size of the wedge was scaled in proportion to the amount of total prey that was accounted for by any given taxon. All taxa listed in the key were trapped by pitchers; taxa that are not visible in any particular plots were either not captured at all by that particular *Nepenthes* taxon, or were not captured in sufficient numbers to be resolved. The minimum level of resolution was in the star plots was four percent of total prey caught. Each star chart contains three concentric circles (colored red). In order of increasing size, these denote prey composition values of 12.5, 25, and 50%, respectively.

To determine whether different *Nepenthes* species specialize on particular prey taxa, we compared estimates of Hurlburt’s *PIE*, using single-factor ANOVA and Tukey’s pairwise comparisons. To test for evidence of niche segregation, we performed null-model analysis using the ‘RA-3’ algo-

rithm within the EcoSim software package to quantify niche overlap using Pianka's index of overlap in resource use (Hurlburt 1971; Pianka 1973; Gotelli & Graves 1996; Gotelli & Entsminger 2007). These methods are outlined in greater detail in Ellison & Gotelli (2009) and Chin *et al.* (2014).

Results

Principal component analyses of the pitcher characteristics demonstrated that, at least in terms of gross pitcher morphology, the pitchers of the hybrid *Nepenthes* are intermediate between those of their parent species (Fig. 2A-C). At Matang, the first two principal components described 79.0% of the variation in the data, while at Serian the corresponding value was 85.5% and at Mersing it was 77.2%. The descriptive statistics for the three PCAs are presented in Table 2. Investigation of the loadings for each PC was inconclusive, with no consistent patterns found across the three study sites. This is not surprising – the parent species differ substantially in pitcher structure, so every pitcher component that was measured contributes significantly to the levels of variation detected, meaning that the likelihood of detecting any consistent trends among pitcher characteristics within and among study sites was low.

In the pitchers of all taxa, ants (Formicidae) were the dominant prey type (Table 1, Figs. 3 & 4). This pattern is typical of the vast majority of lowland *Nepenthes* species studied to date (Moran 1996; Adam 1997; Chin *et al.* 2014; Clarke *et al.* 2014), and appears to indicate that ants are the most important type of prey for these plants. The second most abundant prey taxon varied among sites: Diptera at Matang (Fig. 3a), Termitoidae at Serian (where the *Nepenthes* plants grow in and around closed forest) (Fig. 3b), and a variety of taxa at Mersing (Fig. 4). The prey spectra of the hybrids more or less matched their parent species, indicating that all pitchers within a site were exploiting the same prey, and trapping prey taxa (when resolved ordinal rank) in similar proportions, regardless of pitcher characteristics.

Table 3 presents the results of a series of t-tests performed to compare prey capture between *Nepenthes* species (all species at a study site being treated as a single entity) and hybrids (ditto). At Serian and Mersing, the pitchers of *Nepenthes* species caught greater numbers and diversity of prey, but there were no differences at Matang. These results indicate that pitchers in three of the four hybrid swarms examined were less effective at trapping prey than their parent species. In all but two cases (*N. bicalcarata* at Serian and *N. gracilis* in Johor), prey capture rates per pitcher were very low (Table 1), particularly when compared with the findings of Chin *et al.* (2014). This result is intriguing, as the study undertaken by Chin *et al.* (2014) was run at the same time as the present one using the same sampling methods, yet the prey capture rates appear to be somewhat lower.

There were no significant differences in levels of specialization towards prey among co-occurring *Nepenthes* species and hybrids at Mersing ($F_{4,68} = 2.13$, $P = 0.087$) or Matang ($F_{2,57} = 0.49$, $P = 0.616$), but at Serian there was a significantly higher mean value for PIE in *N. bicalcarata* compared to *N. ampullaria* \times *N. bicalcarata* ($F_{2,52} = 6.00$; $P = 0.005$). The results of the null-model analysis show no evidence for niche segregation at any of the three study site/hybrid-species combinations (Table 4). These results concur with those obtained by Chin *et al.* (2014) for niche overlap analysis for prey that is resolved only to the ordinal rank.

Discussion

Summary of our findings and limitations to the experimental design

The results of our analyses demonstrate that, in terms of the morphological characteristics that we measured, the properties of hybrid *Nepenthes* pitchers do lie somewhere between those of their

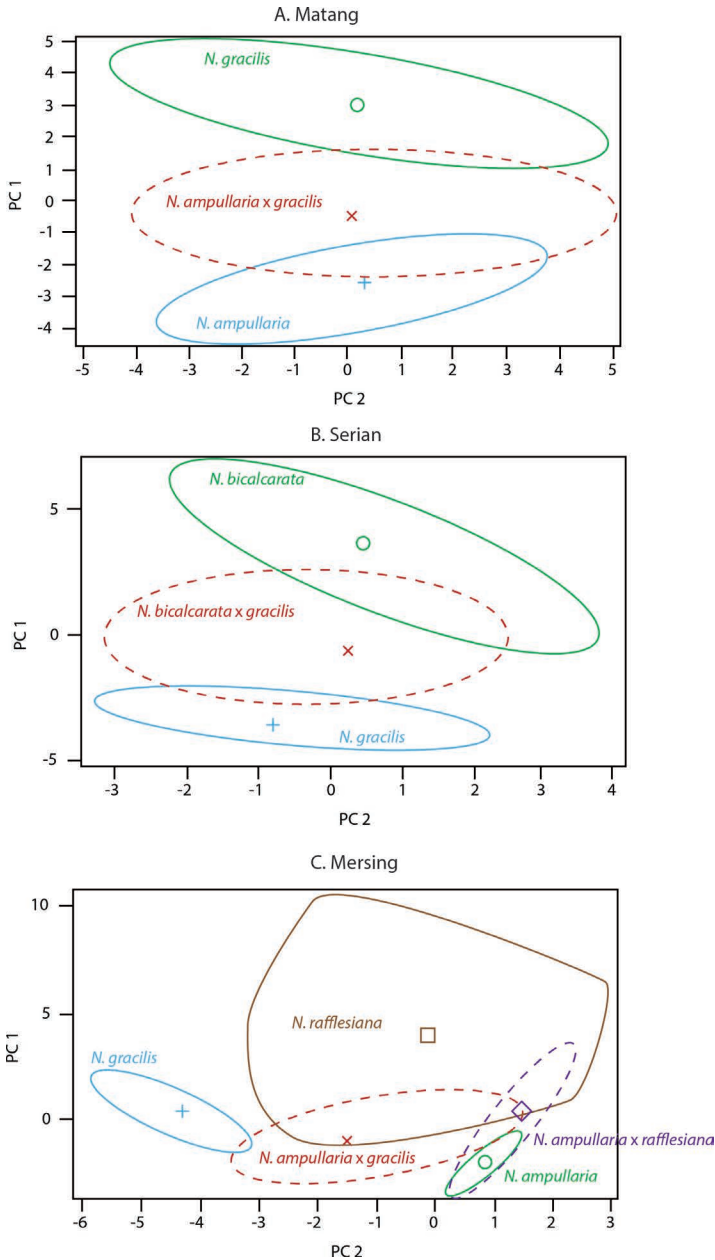


Figure 2: Plots of scores for the first two principal components obtained from PCA of the morphological characteristics of the pitchers of the various *Nepenthes* taxa studied. A. Results for pitchers at Matang. B. Results for pitchers at Serian. C. Results for pitchers at Mersing. For each taxon, within each plot, the mean for PCs 1 & 2 is denoted by a symbol, while the range of values of scores for individual pitchers is described by an ellipse (or polygon, in the case of *N. rafflesiana*). The symbols and ellipses are color-coded to clearly distinguish each taxon.

Table 2. Eigenvectors and contributions of the first three principal components and scores for variables in the principal component analyses for pitcher characteristics at each site.									
Site	Matang			Serian			Mersing		
	PC1	PC2	PC3	PC1	PC2	PC3	PC1	PC2	PC3
Eigenvalue	4.876	3.020	0.686	6.739	1.814	0.531	5.751	1.965	0.611
Proportional contribution	0.488	0.302	0.069	0.674	0.181	0.053	0.575	0.196	0.061
Cumulative contribution	0.488	0.790	0.858	0.674	0.855	0.908	0.575	0.772	0.833
Variable (pitcher characteristic)									
Mouth length	0.153	-0.457	0.337	0.368	-0.018	0.043	-0.375	-0.045	-0.008
Mouth width	-0.005	-0.467	0.310	0.350	-0.139	0.001	-0.277	0.016	0.940
Lid length	0.314	-0.343	-0.289	0.282	0.029	-0.929	-0.385	-0.127	-0.075
Lid width	-0.335	-0.114	0.668	0.361	-0.043	0.155	-0.377	0.001	-0.040
Peristome width	0.399	-0.109	0.014	0.356	0.150	0.114	-0.285	-0.345	-0.064
Pitcher height at rear	-0.296	-0.389	-0.257	0.315	-0.357	-0.030	-0.393	0.029	-0.145
Pitcher height at front	-0.223	-0.413	-0.418	0.156	-0.646	0.118	-0.333	0.107	-0.235
Wax zone length at pitcher front	-0.423	-0.103	-0.123	-0.283	-0.472	-0.044	-0.079	0.680	-0.044
Wax zone length at pitcher rear	-0.428	-0.070	-0.058	-0.294	-0.420	-0.198	-0.154	0.614	-0.008
Capacity	0.328	-0.304	0.038	0.340	-0.102	0.203	-0.340	-0.106	-0.162

parents, and that at two of the three study sites we used, hybrid pitchers caught lower numbers and fewer types of prey (Fig. 2, Table 1). We did not detect evidence of niche segregation with regards to prey capture among any species-hybrid combinations (within study sites), and apart from *N. bicalcarata* at Serian, we did not detect any differences in specialization with regards to prey capture among *Nepenthes* species and hybrids.

There are some important limitations to the quality of the data we obtained, and this impacted upon the results of the subsequent analyses. First, we lacked the expertise to identify the ant species below the rank of suborder (Formicidae). As noted by Chin *et al.* (2014), evidence for niche segregation was only detected when ants were resolved to the rank of genus or species, so although we did not detect evidence for niche segregation in this study, that does not mean that it could not exist. We made no attempt to determine whether the prey spectra of any of the *Nepenthes* taxa we surveyed indicated specialization by the plants towards specific prey type(s) – the objective was purely to compare prey capture patterns among species and hybrids that are exposed to the same arthropod communities. Chin *et al.* (2014) provided an exhaustive review of the limitations to their experimental design, and those limitations also apply to this study. For the sake of brevity, that discussion is not repeated here.

Although the morphological parameters we measured indicate that hybrid pitchers are “the average of those of their parents”, this does not mean that all other aspects of hybrid pitchers necessarily conform to this pattern. In particular, non-physical characteristics of pitchers, such as visual and

Table 3. Comparisons of prey capture patterns between pitchers of <i>Nepenthes</i> species and hybrids at each of the three study sites. * Denotes a significant difference at $P = 0.05$.						
Comparison	Taxa	Number of pitchers	Mean	S.E.	$t_{d.f.}$	P
Matang						
Number of prey types caught	Species	65	0.91	0.10	$t_{50} = 0.30$	0.770
	Hybrids	27	0.85	0.16		
Number of prey items caught	Species	65	2.29	0.43	$t_{29} = 0.83$	0.410
	Hybrids	27	3.78	1.70		
Serian						
Number of prey types caught	Species	53	1.58	0.22	$t_{80} = 2.28$	0.025*
	Hybrids	30	0.85	0.16		
Number of prey items caught	Species	53	44.40	6.10	$t_{57} = 2.50$	0.018*
	Hybrids	30	7.74	1.40		
Mersing						
Number of prey types caught	Species	74	1.18	0.14	$t_{117} = 2.81$	0.006*
	Hybrids	46	0.80	0.12		
Number of prey items caught	Species	74	6.40	1.90	$t_{78} = 2.50$	0.014*
	Hybrids	46	1.63	0.36		

Table 4. Summary of null model analysis of niche overlap in prey utilization. "Observed" is the observed average pair-wise niche overlap. "Expected" is the mean value of average pairwise niche overlap in 10000 randomizations of the resource utilization data. The P value is the upper tail probability of finding the observed pattern if the data were drawn from the null distribution.				
Site	Number of taxa	Observed	Expected	P
Mersing	5	0.98034	0.15049	< 0.001
Serian	3	0.97416	0.17679	< 0.001
Matang	4	0.99261	0.14741	< 0.001

olfactory cues, require detailed investigation to determine how they operate in the parent species, and then how or whether they also operate in hybrids. However, for the purposes of this discussion, we assume that our findings are representative with regards to the physical structure of the pitchers, and we follow the argument of Chin *et al.* (2014) that the pitchers of *N. bicalcarata* and *N. ampullaria* are specialized with regards to nutrient sequestration strategies (Clarke & Kitching 1995; Moran *et al.* 2003).

Implications of our findings

Of the three hybrids examined in this study, *N. bicalcarata* \times *N. gracilis* provides the clearest example of the way in which the specialized characteristics of the pitchers of one parent species (*N. bicalcarata*) are effectively neutralized in hybrid progeny. Four distinctive features of *N. bicalcarata*

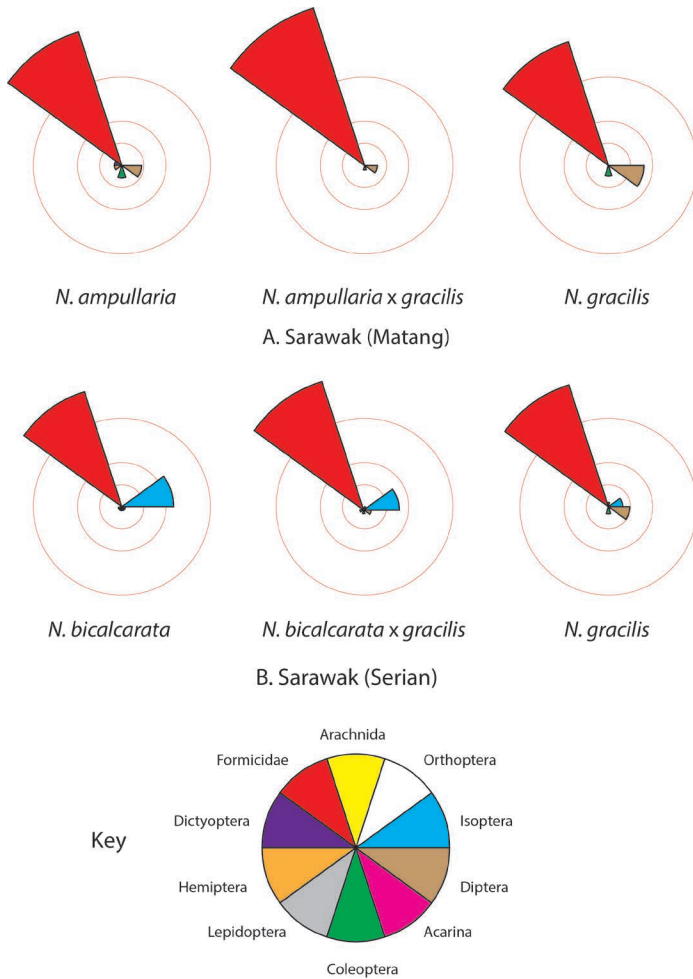


Figure 3: Prey capture patterns (with prey resolved to Order) in *Nepenthes* pitchers at (A) Matang, and (B) Serian, in Sarawak.

pitchers, all of which play important roles in pitcher function, include the large thorns that project down from the base of the pitcher lid (these thorns are actually giant nectaries (Merbach *et al.* 1999)), the wide peristome, the absence of a waxy zone on the inner surfaces of the pitchers, and swollen, hollow pitcher tendrils (Fig. 1B). The thorns provide a source of nectar for *Camponotus schmitzi* ants, which have a mutualistic association with this species (Clarke & Kitching 1995; Bonhomme *et al.* 2010). These ants often wait for prey under the overhanging peristome, and nest in the hollow tendrils. Thus, the physical characteristics of *N. bicalcarata* pitchers demonstrate a high level of specialization towards this mutualism, in which the plant benefits from having its pitchers maintained and prey capture rates enhanced by *C. schmitzi*, in return for providing the ants with food and domicile (Clarke & Kitching 1995; Thornham *et al.* 2012). In *N. bicalcarata* \times *N. gracilis*, the thorns are reduced to small lumps (it is not yet known whether these contain functional nectaries), the peristome is narrow, there is a well-developed waxy zone (which could be difficult for *C.*

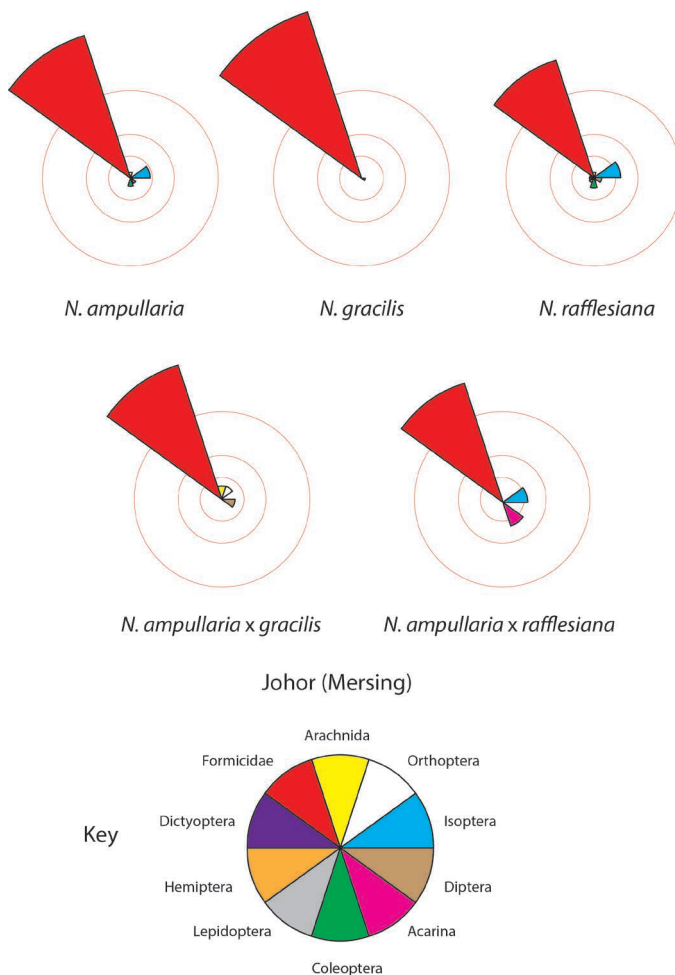


Figure 4: Prey capture patterns (with prey resolved to Order) in *Nepenthes* pitchers at Mersing, in Johor.

schmitzi workers to traverse), and the tendrils lack the cavities which *C. schmitzi* nest in. Therefore, the mutualism between *N. bicalcarata* and *C. schmitzi* cannot exist in this hybrid, because all of the unique traits of *N. bicalcarata* pitchers that facilitate it are either lost or unable to function as they do in *N. bicalcarata*.

On the face of it, *N. bicalcarata* × *N. gracilis* pitchers appear to be more similar to those of its other parent species, *N. gracilis*, in that they have a fairly narrow, cylindrical shape, a clearly defined waxy zone, a narrow peristome and solid tendrils (Fig. 1C). These characteristics are also reasonably representative of the pitchers of many *Nepenthes* species that are assumed to be unspecialized with regards to prey capture, such as *N. mirabilis* and, until recently, *N. gracilis*. However, *N. gracilis* has also been shown to possess a specialized trapping mechanism (Bauer *et al.* 2012b). In this species, the broad pitcher lid has a complex array of wax crystals on its lower surface (similar to those of the waxy zone inside the pitchers) and the thin, flexible nature of the lid enables it to “flick” back and forth when it is gently struck by surrounding objects, or rain

drops. This causes insects that are feeding at nectaries on the lower surface of the lid to be flicked into the pitcher cavity, where they drown and are digested by highly acidic pitcher fluids (Chou *et al.* 2014). By contrast, the lids of *N. bicalcarata* × *N. gracilis* pitchers are thicker and more rigid than those of *N. gracilis* (presumably, this makes them less flexible). It is yet not known whether they possess the complex array of wax crystals on their lower surfaces, or whether they are capable of functioning in the same way as those of *N. gracilis*. If all of the specialized characteristics of *N. bicalcarata* and *N. gracilis* pitchers are also lacking (or operate less effectively) in the hybrid, then by implication, the hybrid pitchers will be unspecialized and are unlikely to be able to obtain supplementary nutrients as effectively as the parent species. This does not mean that hybrid pitchers will fail to trap arthropod prey – virtually any pitfall that contains fluid will trap and retain some arthropods – it simply indicates that the hybrid appears to be less effective at doing so than its parents.

Whether or not hybrids trap enough prey to be able to reproduce effectively remains to be seen, but the evidence from *ad hoc* field observations suggests that for whatever reason, hybrid *Nepenthes* rarely persist in the wild for long periods. There are no known examples of any putative hybrid swarms that show evidence of becoming reproductively stable. There has been some conjecture among biologists that *N. × kinabaluensis* (= *N. rajah* × *N. villosa*) exists as a stable, independent population on Mt. Kinabalu, but there is no evidence of recruitment of new individuals into this swarm via sexual reproduction. Indeed, the only immature plants in the area belong to one of the parent species, *N. villosa*, substantially undermining any arguments for reproductive independence of *N. × kinabaluensis* from at least one of its parent species. Although hybrid *Nepenthes* are fertile, and we have regularly seen pollen collected from hybrid male flowers by a variety of pollinators, examples of fertilization of female hybrid flowers are very rare. At Matang in mid-2010, we observed many female inflorescences of *N. ampullaria* and *N. gracilis* plants, all bearing large numbers of fruits, but only one female inflorescence of *N. ampullaria* × *N. gracilis*, which appeared to bear just one fruit (Fig. 1H). If this pattern is representative, then a lack of recruitment of new individuals (in the absence of introgression with their parents), coupled with apparently low rates of fertilization of female flowers, indicate that while hybrids may be capable of trapping enough prey to produce inflorescences, there may be significant barriers to gene exchange among hybrid plants in the wild, meaning that it is more likely that their pollen will end up on the stigmas of a parent species than on those of another hybrid, or that any pollen that reaches the stigmas of a hybrid inflorescence will probably have come from a parent species as well. Thus, it appears more likely that the primary contribution by hybridization to diversification in *Nepenthes* is to facilitate gene flow between species via introgression.

The potential contribution of introgression to diversification in *Nepenthes* becomes apparent when the role of climate in the distributions of specialized and unspecialized *Nepenthes* are considered. Bauer *et al.* (2012a) and Moran *et al.* (2013) demonstrated that the so-called “dry type” pitcher format (i.e., cylindrical pitcher with a well-developed waxy zone and a narrow peristome) is found throughout the range of the genus, whereas the more specialized “wet type” (i.e., broader pitcher with reduced/no waxy zone and a broad peristome) is largely confined to perhumid habitats in the Sunda region. All of the highly specialized species from Borneo and Sumatra that have been detected to date grow in mixed populations with at least one other species of *Nepenthes*, and usually that species has the dry type pitcher format (C. Clarke, pers. observ.). In general, species whose pitchers conform to the dry type format appear to be less specialized (although there are some notable exceptions, such as *N. albomarginata* and *N. campanulata*), but tolerant of greater variation in environmental parameters than species that utilize the wet type trap format.

In undisturbed habitats, reproductive isolation prevents gene flow between co-occurring species with different trap formats. However, when disturbances occur that are significant enough to disrupt flowering seasons, interspecific gene flow can be facilitated via hybridization followed by introgression. This increases the gene pool of specialized species, which presumably has some negative effect on the effectiveness of its specialization, but highly specialized nutrient sequestration strategies appear to be very sensitive to habitat disturbances (Chin *et al.* 2010), and may break down anyway. Prolonged introgression could effectively “shift” a specialized species away from specialization, and towards a more generalized prey capture strategy. In highly disturbed and unpredictable habitats, this is likely to be beneficial, as undisturbed habitats are the stronghold of specialists, but disturbed and unpredictable habitats are the stronghold of generalists. This may be why we see so many hybrids in recently-disturbed sites in Borneo. If the vegetation at these sites is allowed to recover, the hybrids die out and introgression effectively ceases, but if the disturbances continue, then so does hybridization and introgression. Thus introgression appears to be a mechanism to promote gene flow among *Nepenthes* species during disturbances or periods of environmental unpredictability, making them more resilient, but less specialized.

Conclusion

The role of hybridization in the evolution and diversification of *Nepenthes* remains hypothetical, and this study serves only to show that the amount and variety of prey caught by three common lowland hybrids is less than, or equal to, that of their parent taxa. This could be a consequence of loss (or reduction) of specialized pitcher structures in hybrids that have one or more parent species that target specific types of prey using highly modified pitchers. Detailed manipulative experiments are required to investigate the ecological fitness of hybrid *Nepenthes* in the wild, and we hope that this study will somehow contribute to these.

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