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# Keeping an eye on coloration: ecological correlates of the evolution of pitcher traits in the genus *Nepenthes* (Caryophyllales)

KADEEM J. GILBERT[1*], JOEL H. NITTA[1†], GERARD TALAVERA[1,2] and
NAOMI E. PIERCE[1]

[1]*Department of Organismic and Evolutionary Biology, Harvard University, 26 Oxford St., Cambridge, MA 02138, USA*
[2]*Institut de Biologia Evolutiva (CSIC-Universitat Pompeu Fabra), Passeig Marítim de la Barceloneta, 37, E-08003, Barcelona, Spain*

[†]*Current address: Department of Botany, National Museum of Nature and Science, 4-1-1 Amakubo, Tsukuba, 305-0005, Japan*

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*Nepenthes* is a genus of carnivorous pitcher plants with high intra- and interspecific morphological diversity. Many species produce dimorphic pitchers, and the relative production rate of the two morphs varies interspecifically. Despite their probable ecological importance to the plants, little is known about the selective context under which various pitcher traits have evolved. This is especially true of colour-related traits, which have not been examined in a phylogenetic context. Using field observations of one polymorphic species (*N. gracilis*) and comparative phylogenetic analysis of 85 species across the genus, we investigate correlations between colour polymorphism and ecological factors including altitude, light environment and herbivory. In *N. gracilis*, colour does not correlate with amount of prey captured, but red pitchers experience less herbivory. Throughout the genus, colour polymorphism with redder lower pitchers appears to be evolutionarily favoured. We found a lack of phylogenetic signal for most traits, either suggesting that most traits are labile or reflecting the uncertainty regarding the underlying tree topology. This study highlights ecological correlates of the vast phenotypic diversity of this group of tropical plants. We point to a need for future study examining herbivores of *Nepenthes* and experimental investigations on colour polymorphism.

ADDITIONAL KEYWORDS: altitude – carnivorous plants – coloration – comparative methods – herbivory – intraspecific diversity – *Nepenthes* L. – pitcher plants – plant–animal interactions

## INTRODUCTION

Competition for resources can lead to the divergence of a clade into multiple niches and the evolution of novel morphological features. This can be seen in many plant radiations such as the bromeliads, where species that came to occupy water- and nutrient-limited habitats evolved tightly pressed leaf tanks capable of collecting water and nutrient-rich debris (Benzing & Renfrow, 1974; Givnish *et al*., 2011). In addition to resource limitation and/or competition, plants must also routinely respond to a suite of interspecific interactions. For instance, animal pollinators have a prominent role in shaping floral evolution (e.g. Muchhala & Potts, 2007; Kay & Sargent, 2009; Alcantara & Lohmann, 2011; van der Niet & Johnson, 2012; Anderson *et al*., 2014; Boberg *et al*., 2014; Muchhala, Johnsen & Smith, 2014; Lagomarsino *et al*., 2016). Thus, plant morphological evolution can have multiple, interacting biotic and abiotic drivers. However, disentangling the effects of these various drivers is difficult and has not been achieved for many groups.

Pitcher plants are one such group characterized by an adaptation that is subject to multiple interacting drivers. Their pitchers are modified leaves used to capture and digest animal prey – they are nitrogen-acquiring

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*Corresponding author. Email: kgilbert@g.harvard.edu organs analogous to bromeliad tanks, but are also like flowers in their potential to coevolve with animal visitors. Pitcher plants are thus a useful system to investigate the roles of abiotic and biotic effects on the diversification of an adaptive trait. Here we examine the carnivorous plant genus *Nepenthes* L., the most diverse (> 140 species: Cheek & Jebb, 2014) and widespread family of carnivorous pitcher plants. Its core distribution spans most of Southeast Asia, with a few outlying species in Madagascar to the west and New Caledonia to the east, and ranges in altitude from 0 up to 3520 m a.s.l. (McPherson, Robinson & Fleischmann, 2009). The pitchers of different species are used to capture insects, but additionally may be involved in interactions spanning from commensalism to parasitism and mutualism (e.g. Beaver, 1979; Adlassnig, Peroutka & Lendl, 2011; Bazile *et al*., 2012; Thornham *et al*., 2012; Scharmann *et al*., 2013). Pitchers are morphologically complex, exhibiting an array of traits that are under selection by both biotic and abiotic factors that are difficult to tease apart.

Recent work has elucidated the functional significance of pitcher traits, including the thickness of the peristome, presence of a waxy layer, viscosity of the fluid and digestive gland structure in relation to prey-trapping efficiency (Bonhomme *et al*., 2011; Renner & Specht, 2011; Bauer *et al*., 2012). Although it has not yet been well documented whether interspecific differences can be explained by niche-partitioning (Chin, Chung & Clarke, 2014; but see Peng & Clarke, 2015; Gaume *et al*., 2016), the few known specialist trappers point to the importance of animal visitors to phenotypic evolution of pitchers; this includes traits such as the parabolic structure in *N. hemsleyana* Macfarl. that functions as an echolocation guide for its mutualist bat (Schöner *et al*., 2015; Schöner *et al*., 2017). Despite growing knowledge of the significance of different trapping features to the genus, less than 10% of all species have been the subject of ecological studies detailing their specific prey capture strategies (Clarke & Moran, 2011), and the functional significance of many pitcher traits has yet to be explored.

One potentially important, yet understudied set of traits in pitcher plants are those related to intraspecific polymorphism. Many species produce two distinct pitcher morphologies ('morphs') throughout the lifespan of an individual plant: lower pitchers ('lowers'), which grow gravitropically from plants in the rosette phase and possess winged fringes of tissue ('wings'); and upper pitchers ('uppers'), which grow from plants in the climbing phase, twine onto surrounding vegetation via their tendrils and possess a more streamlined form lacking wings (Jebb and Cheek, 1997; Fig. 1). There is some evidence of prey-partitioning between pitcher morphs (Moran, 1996; Rembold *et al*., 2010; Gaume *et al*., 2016) and differences in the symbiont communities of different pitcher morphs (Clarke, 1997a), but little is known about the evolution or functional significance of pitcher dimorphism.

In addition to shape, many species also have pitchers that vary from red to green, often with discrete colour differences between the lower and upper morphs (Fig. 1 – we hereafter refer to the occurrence of discrete colour differences between pitcher morphs within a plant as 'colour polymorphism'). Despite being a conspicuous feature of *Nepenthes*, pitcher coloration is poorly understood. A few studies have examined the role of red pigmentation as a visual signal in carnivorous plants (Schaefer & Ruxton, 2008; Bennett & Ellison, 2009; Foot, Rice & Millet, 2014; Jürgens *et al*., 2015; El-Sayed, Byers & Suckling, 2016), and several have hypothesized that the contrast of red against a green background of foliage could be attractive, although many insects lack red perception (Briscoe and Chittka, 2001). Red was not found to be a prey attractant in studies with sundews (Foot *et al*., 2014; Jürgens *et al*., 2015; El-Sayed *et al*., 2016), but results have been conflicting in pitcher plants (Schaefer & Ruxton, 2008; Bennett & Ellison, 2009).

As red pigmentation in *Nepenthes* is due to anthocyanins (Kováčik, Klejdus & Repčáková, 2012), which are costly to produce (Gould, 2004), the existence of intraspecific colour polymorphism in pitcher plants is particularly puzzling. More generally, the role of plant anthocyanins as visual signals in flowers and fruits is well understood, but the function of anthocyanins in leaves is less well resolved. Multiple competing, although not necessarily mutually exclusive, hypotheses have been proposed for the role of anthocyanins in leaves (Gould, 2004), the majority of which can be divided into two camps: those that argue that anthocyanins primarily serve a physiological role, vs. those that posit that they are visual signals and are primarily a result of coevolving with herbivores (Archetti & Brown, 2004; Archetti *et al*., 2009). Some potential physiological functions of anthocyanins involve protecting leaves from excess light, including UV shielding and free radical scavenging (Feild, Lee & Holbrook, 2001; Hoch, Zeldin & McCown, 2001; Neill & Gould, 2003), and facilitating nutrient resorption in the context of deciduous colour-changing leaves (Hoch, Singsaas, & McCown, 2003). Additionally, abaxial anthocyanins in understorey plants have been proposed to improve photosynthetic efficiency in the 'back-scatter hypothesis', although there is some experimental evidence against this (Hughes, Vogelman, & Smith, 2008). Considering the coevolution hypothesis, anthocyanins may serve as either direct (Schaefer, Rentzsch & Breuer, 2008; Tellez, Rojas & Van Bael, 2016) or indirect (Page & Towers, 2002; Archetti & Brown, 2004; Karageorgou

& Manetas, 2006; Schaefer & Rolshausen, 2006; Lev-Yadun & Gould, 2008; Archetti *et al*., 2009) defence against herbivores and pathogens.

Here, we seek to better understand the functional significance and diversification of dimorphic traits in pitchers using two complementary approaches: (1) a field study of the polymorphic species *N. gracilis* Korth., exploring the functional significance of intraspecific variation in pitcher traits; and (2) a comparative phylogenetic analysis of species across the genus exploring trait evolution more broadly. This approach should allow us to identify broad patterns across the genus that can be verified in more detail within a particular species.

Our study of *N. gracilis* tests the following hypotheses: (1) red pigmentation promotes prey capture and/or symbiont colonization; (2) red pigmentation increases with increasing light intensity; and (3) red-pigmented pitchers show fewer signs of herbivory. Our comparative phylogenetic study first tests for phylogenetic signal in pitcher traits (Felsenstein, 1985). We then use stochastic character mapping to determine if particular colour states are evolutionarily favoured. Finally, we test for the correlation of pitcher traits with each other and with environmental traits, including habitat type and altitude. Moran *et al*. (2013) found precipitation to be a key factor behind the distribution of the traits they examined (peristome width, wax presence and presence of viscoelastic fluid). Furthermore, previous studies hypothesize that the decreasing availability of ants with altitude (Hölldobler & Wilson, 1990) increases selective pressure for evolving specialized dietary strategies (Clarke *et al*., 2009), which could impact many pitcher traits including coloration and dimorphism. As both precipitation and ant abundance covary with altitude, we analyse the role of altitude as a primary abiotic driver of trait evolution. In addition to altitude, we explore habitat and growth habit as proxies for abiotic drivers of coloration evolution.

## METHODS

### INTRASPECIFIC VARIATION IN *N. GRACILIS*

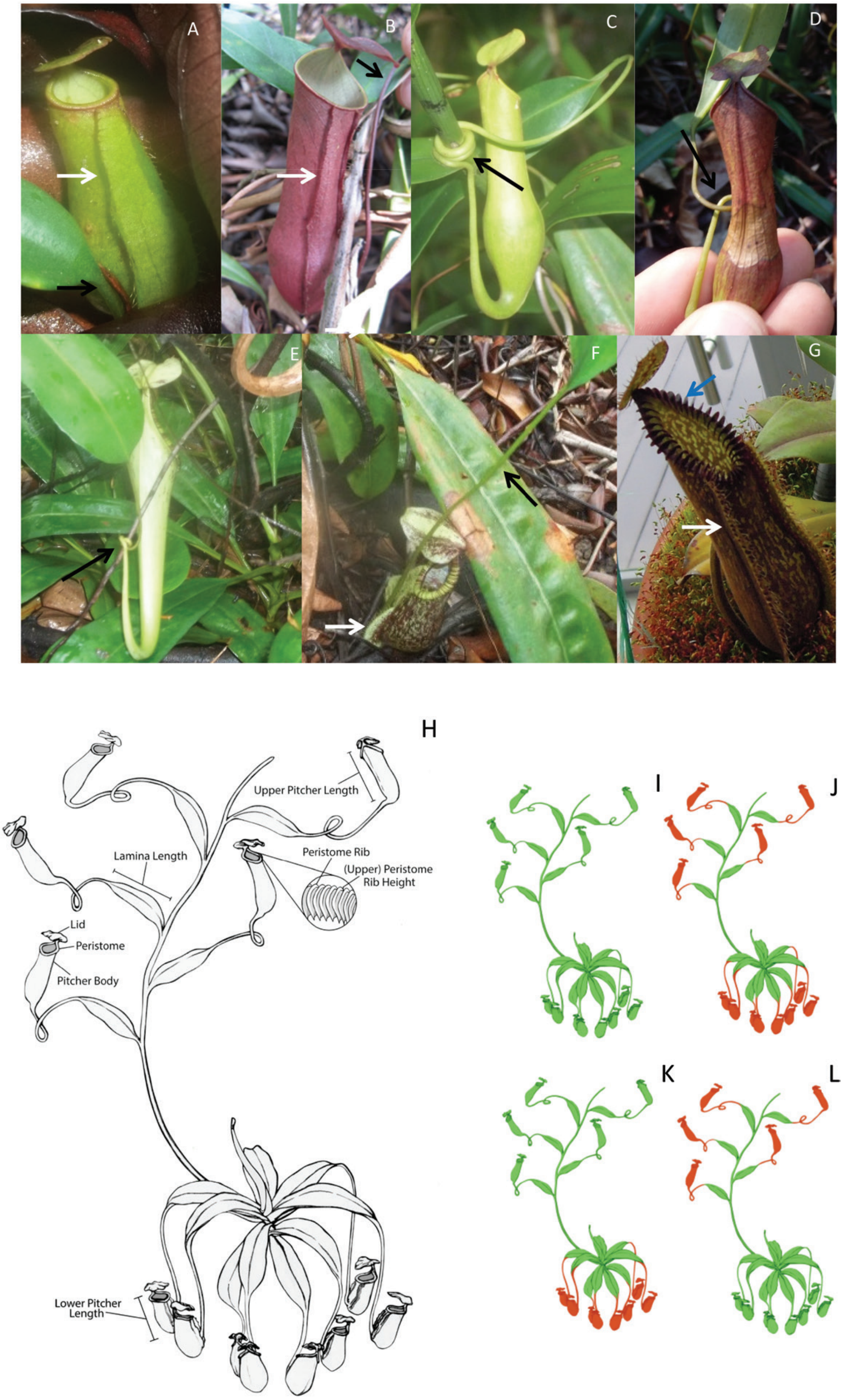
### *Field sites*

Singapore (1.5°N) is aseasonal, with an average annual rainfall of 2340 mm, an average minimum diurnal temperature of 25 °C, an average maximum diurnal temperature of 37 °C, and relative humidity levels generally above 90% in the morning and down to 60% later in the day. The highest point in Singapore is 165 m a.s.l. (Bukit Timah Hill). The natural areas investigated in this study include Kent Ridge Park (1°17′13.00″N, 103°47′10.91″E) and MacRitchie Reservoir Park (1°20′34.99″N, 103°49′47.96″E). Kent Ridge Park is a secondary 'adinandra belukar' forest, dominated by simpoh air (*Dillenia suffruticosa* Martelli) trees. Adinandra belukar type vegetation is characterized by acidic soils (pH 3.3–3.9) and low nitrogen and phosphorous (Chan *et al*., 1997). The MacRitchie Reservoir Park pitcher plants examined grow on the coast of an artificial water reservoir supported by *Ploiarium alternifolium* Melch. and simpoh air trees. *Nepenthes gracilis* is abundant throughout natural areas in Singapore. We chose this species based on its abundance and high level of intraspecific variability. We specifically worked in microhabitats where *N. gracilis* grew isolated from its local congeners (*N. ampullaria* Jack and *N. rafflesiana* Jack).

### *Assessment of insect accumulation rates in different pitcher variants*

In mid-July 2014, pitchers were sampled from two separate areas within Kent Ridge Park separated by about 0.3 km and at one site in MacRitchie Reservoir, which is about 8 km from Kent Ridge. As we could not know how long each pitcher had been open prior to our survey, we needed to 'reset' all of the pitchers in our study sites to be able to compare arthropod colonization rates across pitchers given equal time. We first emptied each pitcher, marked it with a small tag attached to the base of the lamina distal from the pitcher, and then returned to collect its entire fluid contents 1 month after emptying. Arthropods contained in the pitchers were filtered out from the fluid and stored in 100% ethanol prior to being counted and identified according to higher level classification (e.g. order or family depending on the taxon) under a dissecting microscope. We recorded the following characteristics from each sampled pitcher: pitcher morph (upper or lower), pitcher colour (red or green), pitcher condition (healthy or damaged/senescent), the length and width of the pitcher, fluid volume, its distance from the ground, a rank of 'connectedness' (degree to which pitchers formed physical connections with surrounding plants via twining, scored from 1 to 3, with 1 being no connection to other vegetation and 3 being fully twined and well-connected) and the node on which the pitcher occurred.

To determine whether counts of insect prey and symbionts differed significantly between pitchers of differing traits, we performed Poisson regressions using the 'glmer' function in the 'lme4' package (Bates *et al*., 2014) in R 3.3.2 (R Core Team, 2013). We collected from multiple pitchers per plant, so we set plant as a random effect, as well as collection site. We included all examined traits (pitcher colour, pitcher morph, connectedness, pitcher size, and distance from the ground) as fixed effects in one model to account for any correlations among traits. To avoid the potential confounding effects of senescence or increased herbivory, pitchers


A
B
C
D
E
F
G
H
Upper Pitcher Length
Lamina Length
Peristome Rib
(Upper) Peristome Rib Height
Lid
Peristome
Pitcher Body
Lower Pitcher Length
I
J
K
L

that deteriorated in condition over the 1-month period after emptying were excluded from the analysis. We tested for statistically significant differences in numbers of ants, culicid larvae (mosquitoes), non-culicid larvae (all low-abundance dipteran taxa), mites and flying prey items between pitchers that differed in all of the aforementioned measured pitcher characteristics.

### *Assessment of the relationship between pitcher colour and canopy coverage*

In January 2014, for eight arbitrarily selected locations within Kent Ridge Park, we laid out plots approximately 1.5 m in diameter and then exhaustively tallied all of the pitchers within the plots. Based on morph and colour, pitchers were assigned to one of four categories: red lower, red upper, green lower and green upper. We estimated the canopy coverage by photographing the canopy above each plot (pointing upwards from the level of the pitchers at the centre of the plot) using a digital camera (Canon PowerShot ELPH 170IS) and calculating the total area of shade-free space in each image by counting white cells using grids of 625 pixel$^2$ per cell in ImageJ (Rasband, 2012). We tested for a correlation between canopy coverage and the proportion of red pitchers per plot using a linear regression.

### *Assessment of the relationship between pitcher colour and herbivory*

In late January to early February 2016, we tallied pitchers within eight plots in Kent Ridge Park as described above. To test for a relationship between red pigmentation and herbivory in *N. gracilis*, we scored each pitcher within a plot for pitcher type (the four categories of colour and morph described above) and for whether the pitcher exhibited signs of herbivory or pathogen attack. Pitchers were scored as having signs of attack based on the presence of localized spots of discoloured, senescent or missing tissue anywhere on the pitcher body (this was treated as a binary character, so any pitchers lacking such signs were scored as 'not attacked'). We performed a logistic regression using the 'glmer' function in the 'lme4' package (Bates *et al*., 2014) in R 3.3.2 (RCore Team, 2013) to test for a relationship between pitcher colour and signs of attack, including plot as a random effect. We also performed a logistic regression in the same way on the subset of lower pitchers to examine the effect of colour while controlling for morph. To test for a relationship between pitcher morph and signs of attack while controlling for colour, we performed a logistic regression on the subset of green pitchers. The number of red upper pitchers ($n = 1$) was too small to meaningfully compare red uppers and lowers or red and green uppers.

## COMPARATIVE ANALYSIS OF INTERSPECIFIC VARIATION IN *NEPENTHES*

### *Sequence mining and phylogenetic inference*

Previous molecular studies of the genus have utilized different markers: the peptide transferase single copy nuclear gene (*PTR1*: Meimberg & Heubl, 2006), the plastid *trnK* intron (Meimberg *et al*., 2001, 2006; Merckx *et al*., 2015) and the nuclear ribosomal transcribed spacers (*nrITS1-5.8S-nrITS2*: Alamsyah & Ito, 2013; Schwallier *et al*., 2016). Only those studies using *PTR1* and *trnK* shared voucher specimens, so these were the two markers we chose for phylogenetic inference to ensure the taxonomic identity of the specimens was consistent between sequences, especially as the risk of misidentified sequences is a caveat inherent to the use of sequences obtained from a database. While currently available sequence data have proven insufficient to conclusively resolve the phylogeny of *Nepenthes* (Meimberg *et al*., 2001, 2006; Alamsyah & Ito, 2013), they nevertheless provide a working hypothesis with which to begin looking for patterns. Sequences were downloaded from GenBank, resulting in 87 sequences for the ~2500-bp *trnK* plastid gene region, and 40 sequences for the ~1605-bp *PTR1* nuclear gene. We did not use sequences for the pseudogenized copy of the *trnK* gene (Meimberg *et al*., 2006). Our outgroups were *Triphyophyllum peltatum* and *Ancistrocladus abbreviatus*, which both have *trnK* sequences (Meimberg *et al*., 2001). Specimen information and sequences used are summarized in Supporting Information, Table S1. Sequences for each of the genes were aligned separately using MUSCLE (Edgar, 2004) in the Geneious 7.0 platform. To remove ambiguously aligned regions, Gblocks 091 with relaxed parameters

**Figure 1.** (A–D) Photographs showing polymorphism in *Nepenthes gracilis*: (A) green lower pitcher, (B) red lower pitcher, (C) green upper pitcher and (D) red upper pitcher. An (E) upper and (F) lower pitcher of *N. rafflesiana* showing a second example of pitcher dimorphism. Note the difference in tendrils (black arrows) between morphs, which twine in uppers and grow gravitropically in lowers, and note the wings (white arrows) in lower pitchers, which are lacking or reduced in uppers. (G) A lower pitcher of *N. hamata*, indicating the peristome with tall ribs (blue arrow). (H) Diagram of a generalized *Nepenthes* plant with key morphological features labelled. Diagram of plants with (I, J) similar coloration between morphs, (K) redder lower pitchers and (L) redder upper pitchers. Photo credits: A and C–G, K. Gilbert; B, S. Johnson-Freyd. Illustration credit: H–L, Abraham Cone.

(Castresana, 2000; Talavera & Castresana, 2007) was applied to the *trnK* alignment. Best-fitting models for DNA substitution for each marker were selected according to the corrected Akaike information criterion (AICc) in jModeltest v0.118 (Posada, 2008). These resulted in GTR+G for *trnK* and GTR+I for *PTR1*.

An ultrametric tree was inferred using a Bayesian Markov chain Monte Carlo (MCMC) method in the program BEAST v1.8.3 (Drummond *et al*., 2012). A Yule tree prior model and a strict clock were applied (as no definitive fossils of Nepenthaceae are known, no fossil calibration points were used), and two independent chains were run for 10 million generations. Convergence was inspected in Tracer v1.5 and a 10% burn-in was applied to each chain to obtain the final tree.

### *Character matrix*

A character matrix was gathered from species descriptions in McPherson *et al.* (2009), which includes accounts of 125 species and incompletely diagnosed taxa. Using a single source has the advantage of greater consistency in the scoring of characters, in particular those related to colour. Scoring of such traits may be subjective and vary between accounts; furthermore, original species descriptions do not always describe colour variation in depth or provide colour photographs. Another problem with this source is that the information on colour variation within species is based on qualitative descriptions as opposed to quantitative descriptions of the proportions of colour variants within pitcher morphs. Some species have variable coloration, and without data on the proportions of colour variants, both morphs may be described as 'variable', which may mask finer details (i.e. whether the two morphs have different probabilities of being red); however, this still allows us to examine broad patterns. We note that this field guide is not a peer-reviewed source, so wherever possible we have also cross-checked this information against the Jebb & Cheek (1997) *Nepenthes* monograph. We have also included some additional data (peristome width/slope and viscosity) from Bauer *et al*. (2012) for further comparison. Finally, our data are constrained to colours that are found in the visible spectrum. Certain pitcher plant species are known to be strongly reflective and/or absorbing in the UV as well as long wavelengths (e.g. Joel, Juniper & Dafne, 1985; Moran, Booth & Charles, 1999), and the UV in particular may be important in signals involving insects. However, because only a few pitcher plant species have been assessed for their spectral qualities outside the visible, we were unable to include a wider range of wavelengths in our analysis.

In our scoring for colour polymorphism, we scored species either as 'redder lower', 'redder upper' or 'similar coloration'. All of these scores deal specifically with levels of red pigmentation. If a species produces mostly solid-coloured pitchers and the lower pitchers are generally red (to the human eye) and the upper pitchers are generally green (to the human eye), then it was scored as 'redder lower'; the reverse was scored as 'redder upper'. For species with patterning (blotches, spots, stripes or mottles of red/dark pigmentation on the outer pitcher wall), the pitcher morph with denser pigment patterning was considered to be 'redder'. Darker coloured pigmentation was assumed to be the result of increased expression of anthocyanin, so a morph with solid or patterned 'black', 'purple' or 'brown' colour was considered to be redder than a morph with solid or patterned 'red', 'orange' or 'pink' colour. In cases of variation within a pitcher morph, the most commonly observed coloration was used for the comparison. Species with pitcher morphs that are deemed to be generally equivalent for all of the above-described properties were scored as 'similar coloration'. Species where both pitcher morphs exhibit colour variation and both pitcher morphs are described as 'equally variable' were also scored as '1' for 'similar coloration'.

'Lid contrast' and 'peristome contrast' refer to whether the lid/peristome differs in coloration from the pitcher body, for example a green pitcher body with a red lid/peristome or a red pitcher body with a green lid/peristome. The underside of the pitcher lid is generally lighter in colour than the outer wall of the pitcher body, so this did not factor into the scoring of this set of characters. However, in terms of increased pigment relative to the body, a pitcher was scored as having 'lid contrast' based on either the entire lid, the upper surface of the lid or the under surface of the lid – wherever the strongly contrasting red or green coloration is expressed. The contrast scores for lids/peristomes were based primarily on solid colours and any spots or stripes were not considered. Peristome striping was scored as a binary trait, where the trait was scored as present whenever any expression of the trait is reported in a given species. All patterning traits were scored independently for each pitcher morph.

We scored presence/absence of pitcher dimorphism and a related yet distinct trait we refer to as 'reduced lower pitcher production' or 'reduced lowers'. These species still produce both morphs, except that they only produce lowers in young plants and then switch to solely producing uppers, as compared to other species that continue to produce both when mature. Both pitcher dimorphism and reduced lowers were scored as binary.

We scored each of three growth habits (terrestrial, epiphytic, lithophytic) and each of nine habitats (lowland dipterocarp forest, peat swamp, heath forest, montane forest, scrub, cliff, mangrove, seasonal grassland

and degraded – which includes all anthropogenically modified environments) as binary traits, denoting the presence or absence of a species in that habit/habitat.

Species designations follow the taxonomy of McPherson *et al.* (2009). Given this, the Meimberg *et al.* (2001) accession named as *N. anamensis* was scored according to the McPherson *et al.* (2009) account of *N. smilesii*. *Nepenthes xiphioides* and *N. pectinata* were both scored identically to *N. gymnamphora*. Because what Meimberg *et al.* (2001) designates as *N. pilosa* is probably *N. chaniana* (Clarke, Lee & McPherson, 2006), we have relabelled their '*N. pilosa*' sequence as '*N. chaniana* 3' ('*N. chaniana*' and '*N. chaniana* 2' are from Merckx *et al.*, 2015 data).

### *Phylogenetic tests*

All statistical analyses were conducted in R 3.3.2 (R Core Team 2013). We tested for phylogenetic signal in continuous traits with Blomberg's $K$ (Blomberg, Garland & Ives, 2003) and Pagel's lambda (Pagel, 1999) using the 'phylosig' function in the 'phytools' package (Revell, 2012), and in binary traits with Fritz and Purvis's $D$ (Fritz & Purvis, 2010) using the 'phylo.d' function in the 'caper' package (Orme, 2013). To find the number of transitions between states for colour polymorphism we used the 'countSimmap' function using the 'phytools' package; this method is a form of stochastic character mapping and has the advantage of accounting for uncertainty in the underlying topology (Bollback, 2006). To test for the influence of altitude on pitcher traits, we performed a phylogenetic generalized least squares (pgls; for continuous traits) using the 'pgls' function in the 'caper' package and utilized the 'brunch' function (for discrete traits) in the 'caper' package. We used pgls to examine correlations between morphological traits. To test for correlated evolution between colour polymorphism and patterned pitchers, between reduced lowers and colour traits, and between various traits and habitat/growth habit, we used a binary PGLMM (phylogenetic generalized linear mixed model) using the ape package (Paradis *et al.* 2016).

## RESULTS

### INTRASPECIFIC VARIATION IN *NEPENTHES GRACILIS*

At Kent Ridge Park and our site in MacRitchie Reservoir, we collected all the fluid and associated organisms from 83 pitchers of *N. gracilis* (31 individual plants, Supporting Information, Table S2). We counted a total of 822 pitchers in Kent Ridge Park during our January 2014 survey of *N. gracilis* in relation to canopy coverage, and a total of 605 pitchers during our January 2016 survey of *N. gracilis* in relation to herbivory.

There were no significant differences in counts of prey (ants, mites and flying prey) or symbionts (culicids and other larvae) explained by pitcher colour, morph, connectedness, distance from the ground or size (Poisson regression, $P > 0.05$ in all cases, Supporting Information, Table S3), except that pitcher size is positively correlated with number of ants ($P < 0.05$, Table S3).

From our January 2014 survey, we found that lower pitchers were disproportionately more likely to be red-pigmented than upper pitchers (chi-squared test, $\chi^2 = 148.3$, $P < 0.001$; Table S4), showing that *N. gracilis* has 'redder lower' colour polymorphism. We also found a significant positive correlation between the proportion of red pitchers in a site and canopy cover ($r^2 = 0.79$, $P < 0.01$, Fig. 2). From our January 2016 survey, we found that red pitchers were disproportionately less likely to show signs of herbivore or pathogen attack in the field (logistic regression, $P = 0.002$). This result was similar when accounting for pitcher morph by only comparing red and green lowers (logistic regression, $P = 0.002$). However, there was no difference in the likelihood of attack due to pitcher morph, either in the full dataset (logistic regression, $P = 0.72$) or between the green subset of uppers and lowers (logistic regression, $P = 0.47$).

### PHYLOGENETIC INFERENCE

The phylogeny we constructed using the *trnK* and *PTR1* genes (Figs 3, 4) is similar to the phylogenies published by Meimberg *et al.* (2001) and Meimberg & Heubl (2006). The first split within *Nepenthes* separates a clade consisting of *Nepenthes khasiana* and *N. madagascariensis* + *N. masoalensis* from the

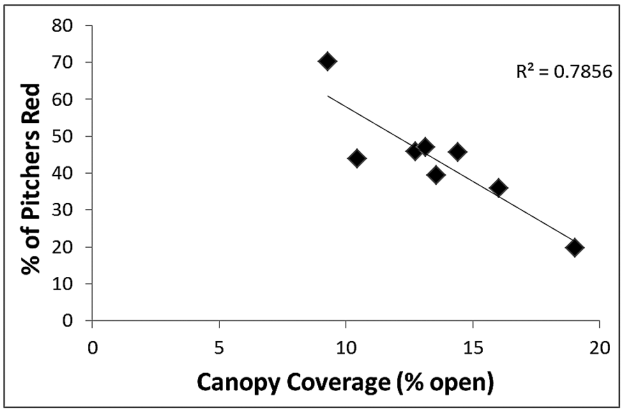


**Figure 2.** Linear regression between canopy coverage and percentage of red pitchers for January 2014 field observations in Kent Ridge Park, Singapore, showing a negative correlation between red pigmentation and light environment. Each point represents a single patch. $P = 0.003$.

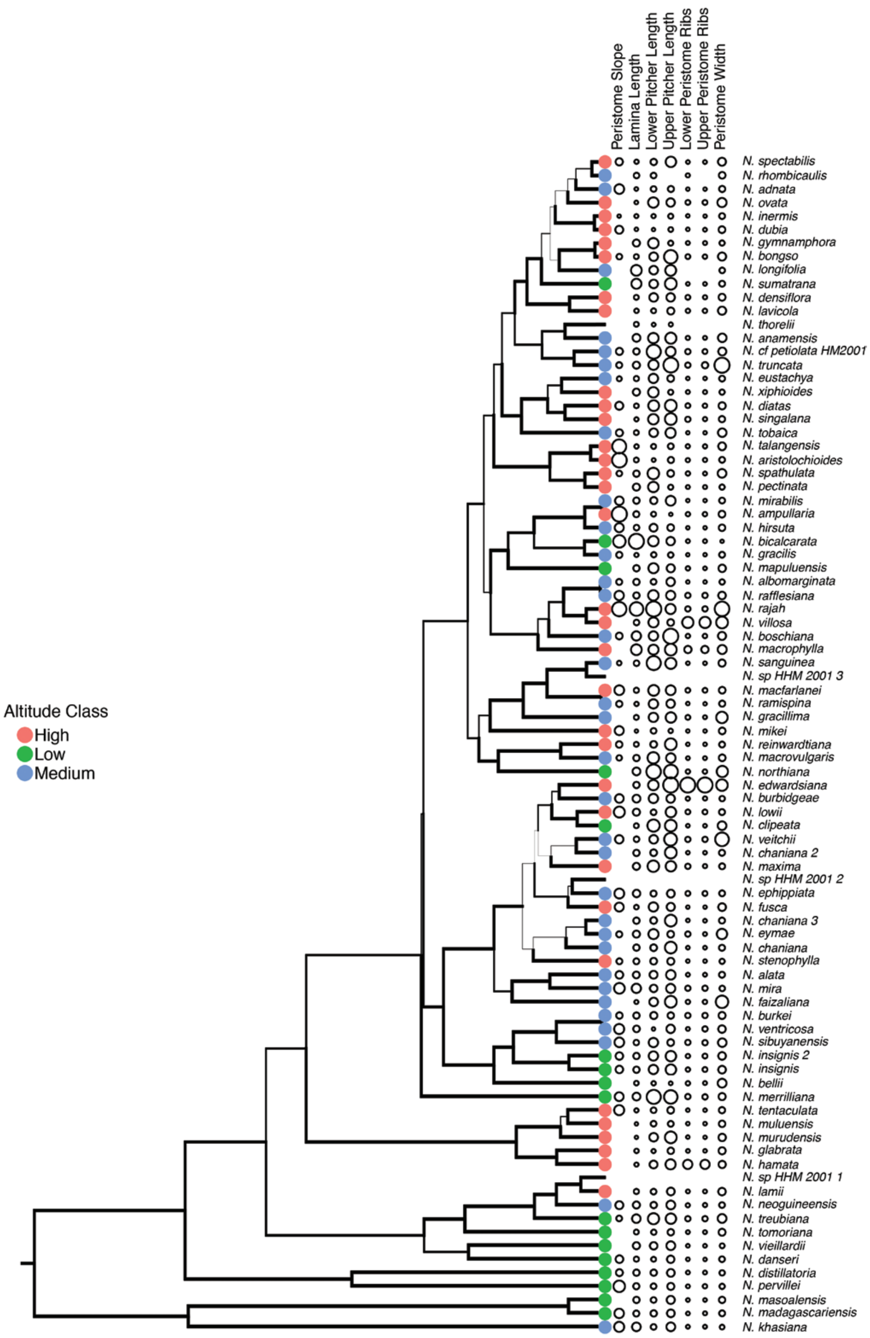


**Figure 3.** Phylogeny displaying topology for the *trnK+PTR1* tree mapped with quantitative morphological traits from McPherson *et al.* (2009) and Bauer *et al.* (2012). Each taxon is placed in an altitude class based on its recorded maximum occurring altitude (McPherson *et al.*, 2009): low (0–1000 m a.s.l.), medium (1001–2000 m a.s.l.) and high (2001–3520 m a.s.l.). The size of the circle icon corresponds to the relative magnitude of that trait for the given taxon. Line weights in the phylogeny are proportional to the posterior probability values of its subtending node.

remaining species, which are then split into a clade consisting of *N. pervillei* + *N. distillatoria* and the rest of *Nepenthes*. These two smaller clades include the 'outlying' species from the Western limits of the genus' range (India, Madagascar, the Seychelles and Sri Lanka), which have appeared in a similar position in all of the phylogenies published thus far (often referred to as 'basal' species in previous studies, e.g. Meimberg *et al.*, 2001, 2006; Alamsyah & Ito, 2013). Branch lengths become much shorter and

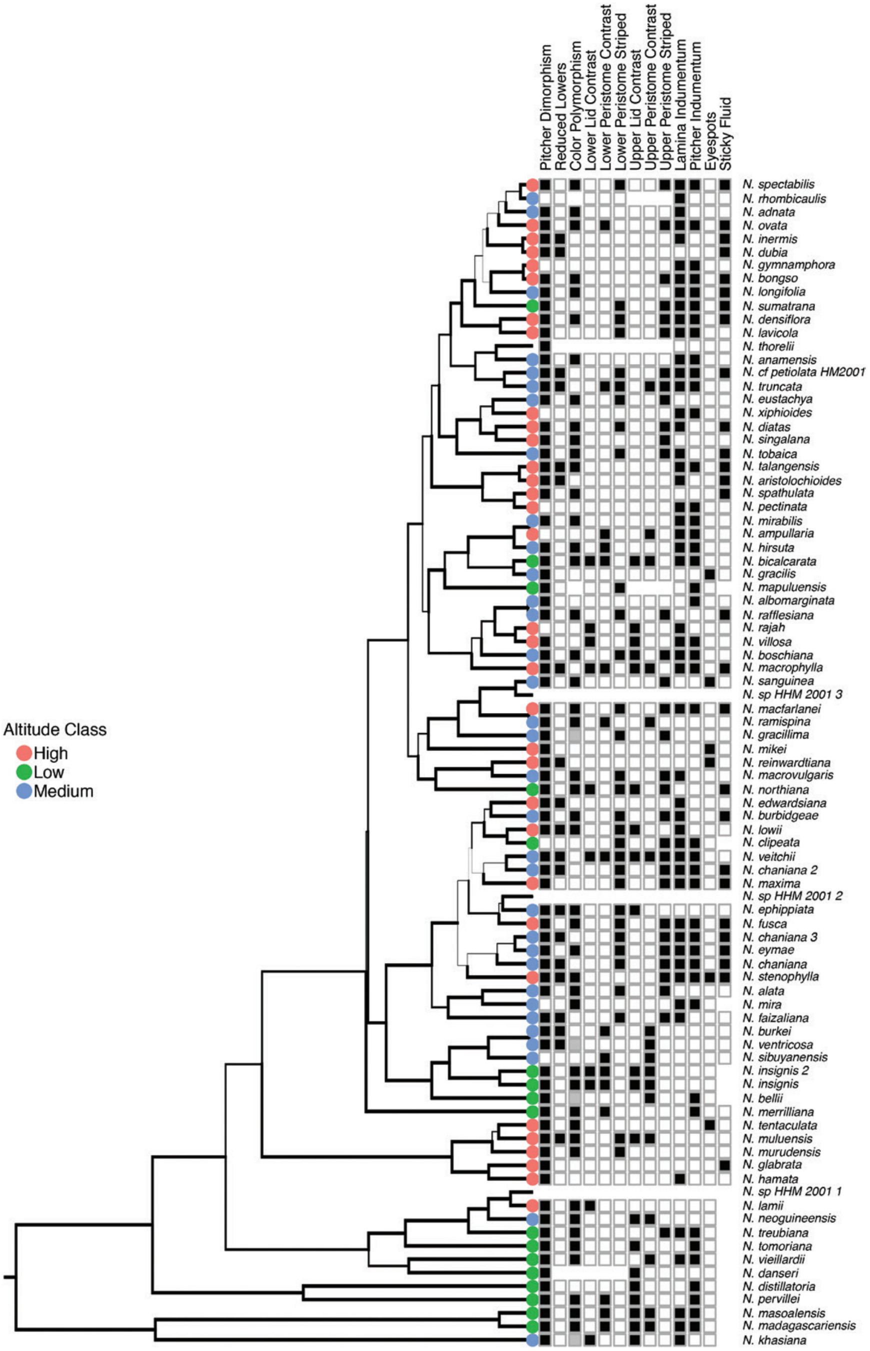


**Figure 4.** Phylogeny displaying topology for the *trnK+PTR1* tree mapped with qualitative morphological traits from McPherson *et al*. (2009). Each taxon is placed in an altitude class based on its recorded maximum occurring altitude (McPherson *et al*., 2009): low (0–1000 m a.s.l.), medium (1001–2000 m a.s.l.) and high (2001–3520 m a.s.l.). Where information is available, the presence of the trait is represented by a black square and the absence of the trait by an open square. Colour polymorphism is the exception as the sole non-binary qualitative trait. Here the three states are: similar coloration between morphs (open square), redder lower pitchers (black square) and redder upper pitchers (grey square). Line weights in the phylogeny are proportional to the posterior probability values of its subtending node.

less well resolved for the numerous species from the Southeast Asian centre of distribution. Within this large Southeast Asian clade, a clade consisting mostly of Papuan species is sister to the remaining species. As with previous studies, however, several nodes are poorly resolved, particularly within the aforementioned large Southeast Asian clade containing species from Sundaland, the Philippines and western Wallacea.

### PHYLOGENETIC SIGNAL

None of the quantitative traits we examined exhibits significant phylogenetic signal, neither with Pagel's lambda nor with Blomberg's *K* (Table 1). None of the binary traits we examined exhibits significant phylogenetic signal (Table 2), except for lower peristome stripes (probability of random distribution = 0.003). The lack of significant signal in the majority of these traits may suggest evolutionary lability in *Nepenthes* pitcher evolution or may equally plausibly be attributed to the lack of topological resolution inferred from the currently available genetic data.

**Table 1.** Phylogenetic signal in continuous (quantitative) traits using Pagel's lambda (Pagel, 1999) and Blomberg's *K* statistic (Blomberg *et al*., 2003)

| Trait | lambda | *K* |
|---|---|---|
| Lamina length | 0 ($P = 1$) | 0.11 ($P = 0.4$) |
| Lower pitcher length | 0.22 ($P = 0.31$) | 0.16 ($P = 0.02$) |
| Upper pitcher length | 0 ($P = 1$) | 0.09 ($P = 0.48$) |
| Lower peristome rib height | 0 ($P = 1$) | 0.13 ($P = 0.38$) |
| Upper peristome rib height | 0 ($P = 1$) | 0.13 ($P = 0.33$) |
| Peristome width | 0 ($P = 1$) | 0.18 ($P = 0.02$) |
| Peristome slope | 0 ($P = 1$) | 0.06 ($P = 0.81$) |

*P* values are in parentheses. Lamina length, upper/lower pitcher length and upper/lower peristome rib heights are values from McPherson *et al*. (2009). Peristome width (peristome width values corrected for pitcher length) and peristome slope (the length of the inward sloping portion of the peristome) are values taken from Bauer *et al*. (2012) for comparison. Values significant at the Bonferroni-corrected alpha value of 0.00625 are indicated by an asterisk. Significant values indicate a trait with phylogenetic signal.

### STATE SWITCHES IN COLOUR POLYMORPHISM

Our analysis of state switches in the colour polymorphism trait yielded 'redder lowers' as the state with the longest evolutionary residence time, followed by 'similar coloration' and the shortest time for 'redder uppers' (proportion of time spent in state being 0.52, 0.31 and 0.17, respectively). Switches occurring between 'redder lowers' and 'similar coloration' are more numerous than any of the switches involving 'redder uppers'. Switches away from 'redder uppers' are more numerous than switches to 'redder uppers'. Switches between 'redder lowers' and 'redder uppers' are more numerous than those between 'similar coloration' and 'redder uppers'. Overall, together with the likelihood that 'redder lowers' is ancestral, these trends imply that 'redder lowers' is the default state and 'redder uppers' is evolutionarily disfavoured relative to the other two states (Fig. 5).

### CORRELATIONS WITH ALTITUDE AND BETWEEN MORPHOLOGICAL TRAITS

None of the quantitative or binary traits we examined exhibits a significant relationship with altitude (pgls,

**Table 2.** Phylogenetic signal in binary traits using Fritz and Purvis's *D* statistic

| Trait | Estimated *D* | prob_random | prob_brownian |
|---|---|---|---|
| Pitcher dimorphism | 1.13 | 0.567 | 0.056 |
| Reduced lower pitchers | 0.22 | 0.007 | 0.333 |
| Similar coloration | 1.14 | 0.674 | 0.003* |
| Redder lowers | 0.93 | 0.401 | 0.008 |
| Redder uppers | 0.53 | 0.251 | 0.383 |
| Lower lid contrast | 0.57 | 0.144 | 0.165 |
| Lower peristome contrast | 0.68 | 0.137 | 0.046 |
| Lower peristome stripes | 0.13 | 0.003* | 0.393 |
| Upper lid contrast | 0.20 | 0.005 | 0.32 |
| Upper peristome contrast | 0.46 | 0.041 | 0.126 |
| Upper peristome stripes | 0.53 | 0.042 | 0.077 |
| Lamina indumentum | 0.78 | 0.199 | 0.009 |
| Pitcher indumentum | 0.85 | 0.277 | 0.009 |
| Eyespots | 0.74 | 0.243 | 0.106 |

Given is the estimated *D* statistic for each trait (Fritz & Purvis, 2010) as well as the probability that the trait is randomly distributed in the phylogeny (for a true random distribution *D* not significantly different from 1), and the probability that the trait is distributed according to a Brownian pattern (*D* not significantly different from 0). The extreme values for the *D* statistic are –2.4 for clumped and 1.9 for overdispersed. The scoring of these binary traits derived from McPherson *et al*. (2009) is described in the Methods. 'Similar coloration', 'redder lowers' and 'redder uppers' are all elements of colour polymorphism converted to binary. Values significant at the Bonferroni-corrected alpha value of 0.0035 are indicated by an asterisk.

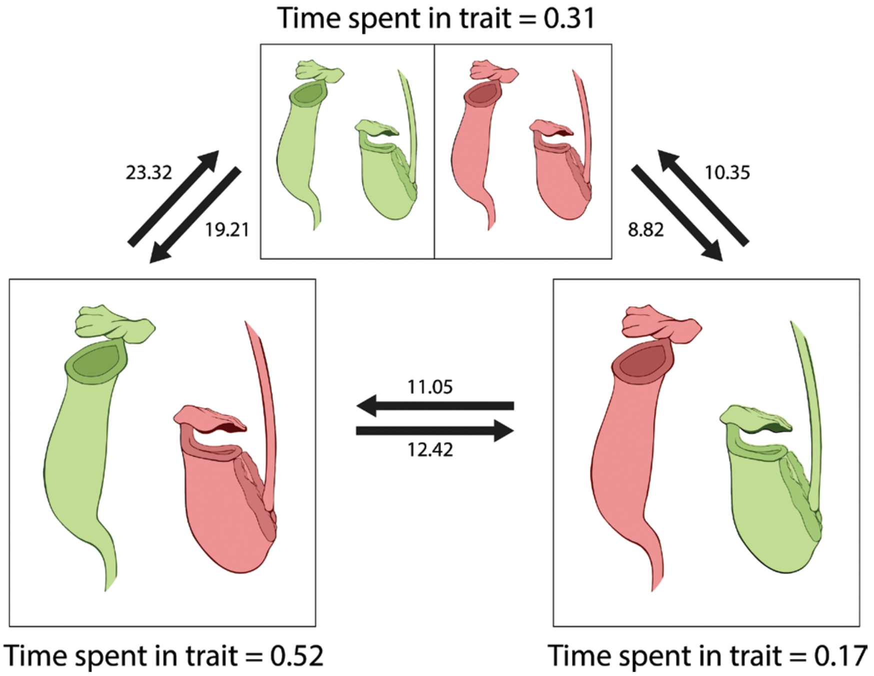


**Figure 5.** Evolutionary pathways of colour polymorphism. Illustrated are the three states of colour polymorphism we scored (from top, anticlockwise): similar coloration between pitcher morphs, lower pitchers more red-pigmented and upper pitchers more red-pigmented. Arrows show direction of state change. Numbers above arrows represent the frequency of that transition in our character-mapped phylogeny. Note that the majority of transitions initiate from the 'redder lowers' state. Illustration credit: Abraham Cone.

Table 3). Lamina length increases with lower pitcher length (pgls, $P < 0.001$, Table 3) and lower peristome rib height increases with upper peristome rib height (pgls, $P < 0.0001$, Table 3).

### TESTS OF CORRELATED EVOLUTION: COLOUR POLYMORPHISM, REDUCED LOWERS AND HABIT/HABITAT

We found no significant correlated evolution between colour polymorphism and pitcher dimorphism, reduced lower pitchers or patterned pitchers (binary PGLMM, $P > 0.05$ in all cases; Supporting Information, Table S5). The 'reduced lowers' trait is positively correlated with the 'similar coloration' trait (binary PGLMM, correlation estimate = 1.70, $P$ = 0.003; Table S6) and negatively correlated with the 'redder lowers' trait (binary PGLMM, correlation estimate = −1.72, $P$ = 0.003; Table S6). Colour-related traits in general show no significant correlations with growth habit or habitat (binary PGLMM, $P > 0.05$ in all cases; Table S7). There is a significant positive correlation between reduced lowers and epiphytism (binary PGLMM, correlation estimate = 5.90, $P$ = 0.002; Table S7).

## DISCUSSION

Species of *Nepenthes* represent a plant radiation with high morphological and ecological diversity in their pitchers. We took two approaches to evaluating the ecological drivers of dimorphism- and colour-related morphological traits in *Nepenthes*: a field study of the polymorphic species *N. gracilis* and comparative phylogenetic analysis across the genus. Our field studies of *N. gracilis* showed the potential importance of light environment and herbivore pressure to colour polymorphism, with lower pitchers disproportionately more likely to be red-pigmented than upper pitchers (Supporting Information, Table S4), and the proportion of red pitchers in a site significantly correlated with canopy cover (Fig. 2). Our comparative analysis further showed that redder lower pitchers may be evolutionarily favoured across the genus. We found little evidence supporting that altitude, growth habit or habitat are key drivers of the traits we examined (Table S7). We further discuss our results below.

### PITCHER DIMORPHISM

We found no evidence that dimorphism is correlated with altitude (Table S7), contrary to our expectation

**Table 3.** Correlations among quantitative traits using phylogenetic generalized least squares

| | Min altitude | Max altitude | Altitudinal range | Lamina length | Lower pitcher length | Upper pitcher length | Lower peristome rib height |
|---|---|---|---|---|---|---|---|
| Min altitude | **1** | – | – | – | – | – | – |
| Max altitude | **0.53**<br>(2.04E–7*) | **1** | – | – | – | – | – |
| Altitudinal range | **–0.47**<br>(8.40E–6*) | **0.41**<br>(1.00E–4*) | **1** | – | – | – | – |
| Lamina length | **–0.25**<br>(0.0224) | **–0.25**<br>(0.0245) | **0.04**<br>(0.7133) | **1** | – | – | – |
| Lower pitcher length | **–0.12**<br>(0.2860) | **–0.19**<br>(0.0884) | **–0.08**<br>(0.4938) | **0.37**<br>(5.27E–4*) | **1** | – | – |
| Upper pitcher length | **–0.03**<br>(0.7656) | **–0.28**<br>(0.0109) | **–0.30**<br>(0.0062) | **0.19**<br>(0.0789) | **0.27**<br>(0.0152) | **1** | – |
| Lower peristome rib height | **0.29**<br>(0.0081) | **0.33**<br>(0.0028) | **0.04**<br>(0.7544) | **–0.13**<br>(0.2325) | **0.26**<br>(0.0207) | **0.03**<br>(0.8004) | **1** |
| Upper peristome rib height | **0.26**<br>(0.0204) | **0.30**<br>(0.0070) | **0.07**<br>(0.5102) | **–0.11**<br>(0.3344) | **0.12**<br>(0.2532) | **0.08**<br>(0.4632) | **0.95**<br>(0.0000*) |

Pearson's correlation coefficients are in bold type, and $P$-values in parentheses. An asterisk indicates statistical significance at a Bonferroni-corrected alpha level of 0.0017.

that dimorphism would be lost with the decreasing availability of ants at higher altitudes. We also examined situations in which the production of lower pitchers is reduced. We expected this trait to increase with altitude, but again found no significant relationship (Table S7). However, we found that the evolution of reduced lower pitchers is positively correlated with the evolution of epiphytism (Table S7), possibly reflecting that upper pitchers are better suited to an arboreal environment than lower pitchers. Interestingly, only one of the species in our dataset is a strict epiphyte – the rest grow terrestrially as well – so reduced lowers may be a means of entering an epiphytic niche rather than a consequence of becoming epiphytic.

We found no difference in the rate of prey capture between upper and lower pitchers of *N. gracilis* in our observations of this species, and no evidence for partitioning of crawling and flying insects between morphs (Supporting Information, Table S3). This is consistent with the results of Gaume *et al.* (2016), who examined prey capture in seven sympatric Bornean taxa with morphological differences, where *N. gracilis* showed far less difference in prey composition of upper and lower pitchers than the other species examined. This shows that while dimorphism may have a pronounced ecological role in some species, this pattern is not universal throughout the genus.

### COLOUR POLYMORPHISM

We tested three hypotheses regarding the function of red pitchers in *N. gracilis*: (1) red coloration acts as a visual signal to prey and symbionts; (2) red pigmentation protects pitchers from excess solar radiation; and (3) red pigmentation is related to defence.

We found no support for our first two hypotheses: red and green pitchers did not differ in their prey capture rates in *N. gracilis* (Table S3), and red pitchers were significantly less common in areas with greater sun exposure (Fig. 2). The lack of difference in prey capture between red and green pitchers makes sense from the perspective of insect vision: ants, the main prey items, lack an ability to perceive red (Briscoe & Chittka, 2001). Our finding on sun exposure is the opposite from what we would predict if the anthocyanins in red pitchers function primarily to protect against UV. This does not necessarily rule out all possible physiological functions (e.g. protection against sun flecks; Gould *et al.*, 1995), but pitchers are also less photosynthetically active than the laminae (Pavlovič, Masarovičová & Hudák, 2007; Pavlovič *et al.*, 2009; Adamec, 2010a, b), further diminishing the likelihood of a photosynthetic function. However, our observations were consistent with our third hypothesis, that red pigmentation is related in some way to defence.

Defence is probably prioritized more as plants become more nutrient-stressed or energy-limited (Gianoli, 2015). Moran & Moran (1998) showed that red coloration in *N. rafflesiana* can be induced by nutrient stress. In our study, red pitchers were more likely to occur in the shade, and showed significantly fewer signs of herbivory than green pitchers (logistic regression, $P < 0.01$). Thus, pigmentation could play a defensive role in *N. gracilis*, possibly an indication that red pitchers are more chemically well defended (Menzies *et al*., 2016), or less nutritionally valuable, and/or that the coloration defends against herbivores via crypsis (Fadzly *et al*., 2009, 2016; Klooster, Clark & Culley, 2009; Fadzly & Burns, 2010; Niu *et al*., 2014), which is plausible considering that lowers often grow in reddish-brown leaf litter while uppers tend to grow embedded in green foliage (K. Gilbert, pers. obs.). The greater likelihood for lowers to be red compared to uppers also supports the defence hypothesis, as a climbing habit reduces herbivore pressure (Gianoli, 2015). The results of our phylogenetic analyses suggest that the selection for redder lowers we see in *N. gracilis* may be generalizable to the genus as a whole. Not only is the number of clades with redder lowers much greater than that of those with redder uppers, but the 'redder uppers' state has the lowest residence time in our state switch analysis (Fig. 5), suggesting that pigmented upper pitchers are generally not evolutionarily favoured.

Another line of evidence for the putative role of herbivory in shaping colour polymorphism is that the evolution of reduced lowers is associated with the loss of colour polymorphism and a shift away from 'redder lowers' (Supporting Information, Table S6). A lessened investment in producing lowers could conceivably lead to a lessened investment in pigmenting them. Alternatively, as species with reduced lowers have a tendency towards epiphytism, the two morphs may experience more similar environmental conditions than usual, leading to similar coloration. It is notable that epiphytes completely avoid the relatively higher herbivore pressure of terrestrial areas (Gianoli, 2015), so the defensive role of pigmentation would be relaxed. When both pitcher morphs are red in epiphytes, this could be due to a stronger signalling role (see *N. macrophylla* in Moran *et al*., 2012).

### The evolution of contrasting colour patterns within pitchers

In addition to examining colour polymorphism, we explored the evolution of interspecific diversity in contrasting colour patterns, which include a striped peristome, contrast between the colour of peristome and that of the pitcher body, and contrast between the colour of the pitcher lid and pitcher body. This kind of patterning seems likely to play a role in signalling to visually oriented animals that can distinguish between red and green, and a contrasting pattern has already been shown to be important in signalling to vertebrate visitors in coprophagous species (*N. lowii*, *N. rajah* and *N. macrophylla*; Moran *et al*., 2012). More generally, pitcher contrast may be important to anthophilous insects as well (*N. rafflesiana*; Moran *et al*., 1999). We found more origins of peristome and lid contrast in upper pitchers than in lower pitchers (Fig. 4), possibly because upper pitchers tend to be in higher light environments that could make such patterns more effective in signalling.

### Correlations between altitude, habit, and habitat and morphological evolution

The potential ecological drivers we explored in our comparative analysis include altitude, growth habit and habitat. None of the quantitative traits we examined correlates significantly to altitude in our phylogeny (Table 3). However, the trend of increasing lower and upper peristome rib heights in relation to maximum altitude is compelling given the wetness-dependent function of the peristome (Bohn & Federle, 2004; Bauer & Federle, 2009), which is favoured in climates with greater precipitation (Moran *et al*., 2013; Schwallier *et al*., 2016). As precipitation increases with altitude, the trend of peristome rib height increasing with altitude also makes sense. Altitude, growth habit and habitat are all proxies for multiple abiotic factors, so our inability to find significant results for most of our morphological traits using these environmental traits could mean that abiotic factors are generally less important to pitcher evolution than biotic factors, or that our metrics do not accurately capture enough relevant environmental variables.

## CONCLUSIONS

Although much remains to be learned about functional diversity of *Nepenthes* pitchers in relation to diet and prey capture, even less emphasis has been placed on the adaptations used by the plant to deter its own enemies. Our analysis of *Nepenthes* pitcher coloration indicates that herbivory may play a role in maintaining pitcher colour polymorphism, and should be explored further experimentally. Herbivory is an understudied subject in *Nepenthes*, with few publications directly addressing herbivores that attack *Nepenthes* (Clarke, 1997b; Merbach *et al*., 2007; Bauer, Rembold & Grafe, 2016). More generally, the role of anthocyanins as a herbivore defence remains unresolved (Menzies *et al*., 2016), so an improved understanding of the influence of herbivores on pitchers' complex pigmentation strategies may yield novel insights into the broader use of red coloration in leaves – and such questions require polymorphic species as models (Gould *et al*., 2000; Menzies *et al*., 2016). The unique nutritional challenges of carnivorous plants in general (Givnish *et al*., 1984; Juniper, Robins

& Joel, 1989) add weight to the importance of herbivore defence in their ecology; and the biphasic life history of climbing *Nepenthes* emphasizes how environmental context interacts with the potential defensive role of anthocyanins, as evidenced by the prevalence of redder lowers. It is our hope that this study will serve both as a review of the current state of knowledge of *Nepenthes* diversity and stimulate future phylogenetic explorations of this unique plant group.

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## SUPPORTING INFORMATION

**Table S1.** List of taxa with GenBank accession numberss used in phylogenetic reconstructions.
**Table S2.** Summary of collections of pitcher infauna for Singapore, summer 2014.
**Table S3.** Results of Poisson regressions conducted on *Nepenthes gracilis* pitcher infauna counts.
**Table S4.** Contingency table for field survey of *Nepenthes gracilis* colour polymorphism.
**Table S5.** Results of test of correlated evolution (binary PGLMM) between colour polymorphism (converted to binary state, presence or absence of 'similar coloration') and dimorphism, reduced lower pitchers, and the six colour pattern-related traits: upper and lower lid contrast, upper and lower peristome contrast, and upper and lower peristome.
**Table S6.** Results of test of correlated evolution (binary PGLMM) between reduced lower pitcher production trait and dimorphism, three binary colour polymorphism traits (presence/absence of 'similar coloration', 'redder lowers' and 'redder uppers'), and the six colour pattern-related traits: upper and lower lid contrast, upper and lower peristome contrast, and upper and lower peristome stripes.
**Table S7.** Select results of tests of correlated evolution (binary PGLMM) against habit (terrestrial/epiphyte/lithophyte) and habitat (dipterocarp forest, peat swamp, heath forest, montane forest, scrub, cliff, mangrove, seasonal grassland and degraded).

## SHARED DATA

Data deposited in the Dryad digital depository (Gilbert et al., 2017).