

Title: Convergence in carnivorous pitcher plants reveals mechanism for composite trait evolution

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Abstract:

20 Composite traits involve multiple components that, only when combined, gain a new synergistic function. Thus, how they evolve remains a puzzle. We combined field experiments, microscopy, chemical analyses and laser Doppler vibrometry with comparative phylogenetic analyses to show
25 that two carnivorous *Nepenthes* pitcher plant species convergently evolved identical adaptations in three distinct traits to acquire a new, composite trapping mechanism. Comparative analyses suggest that this new trait arose convergently via ‘spontaneous coincidence’ of the required trait combination, rather than directional selection in the component traits. Our results indicate a plausible mechanism for composite trait evolution and highlight the importance of stochastic phenotypic variation as a facilitator of evolutionary novelty.

30 **Short Title:** Mechanism for composite trait evolution

One sentence summary: Evolution of a composite trapping mechanism via spontaneous coincidence of highly variable component traits.

Main Text:

Composite traits consist of multiple independent components which, in combination, gain a novel synergistic function. For example, bird wings combine adaptations of morphology, bone anatomy, and skin appendages to convert a forelimb into an aerofoil (5), and independent modifications of shape (6), coloration (7), and biosynthetic pathways (8) underpin the insect-trapping leaves of carnivorous plants. Explaining the origin of composite traits has puzzled biologists since Darwin (1-4), as it requires coordinated evolution of multiple independent components (9, 10). Thus, additional factors such as phenotypic plasticity (11) have been proposed to play a role in composite trait evolution. Plasticity *sensu lato* includes both induced phenotypic variation, expressed by the same genotype in response to different environments (plasticity *sensu stricto*), and stochastic phenotypic variation, expressed by the same genotype under identical conditions (12-14). Neither type is mediated by genetic differences, but rather by epigenetic modifications which, at least in plants, may be heritable (15, 16). In addition to genetic variation, plasticity *sensu lato* can contribute significantly to the total phenotypic variation under selection (17); however, empirical data demonstrating its role in trait evolution are scarce (18).

The evolution of any given trait is the result of the combined effects of natural selection, where traits are shaped by adaptive pressures and tend to change in a specific direction over time (determinism), and random events and processes, such as genetic drift or chance mutations (stochasticity), in the context of the evolutionary history (contingency) of the trait (28). Here, we report convergence in a complex, composite trapping mechanism in two carnivorous pitcher plant species, and present a plausible mechanism for its evolution, facilitated by high stochastic phenotypic variation enabling the spontaneous coincidence of a new beneficial trait combination.

Springboard trapping – a composite trait with three independent components

Pitcher plants use cup-shaped leaves (Fig. 1A, G) with slippery surfaces to trap insect prey (19). The roof-like pitcher lid is not typically involved in trapping; however, in Southeast Asian *Nepenthes gracilis* it acts as a rain-actuated ‘springboard’ that catapults insects into the fluid-filled trap below (Fig. 1D, movie S1, 34). We discovered that *Nepenthes pervillei*, endemic to the Seychelles and separated from *N. gracilis* by 4,000 km of ocean, uses the same springboard mechanism (Fig. 1J, movie S2).

Springboard trapping requires three independent components: (i) a horizontal lid that accelerates preys into the pitcher, (ii) a moderately slippery underside that allows insects to access the still lid, but not withstand a drop impact, and (iii) a pivoting lid that acts as a torsion spring (20). Each trait only gains a trapping function in the presence of the other two (34).

We first tested whether *N. gracilis* and *N. pervillei* evolved identical adaptations in all three component traits (21). Then, we assessed the same traits in 55 *Nepenthes* species (Table S1), corresponding to roughly one-third of the genus and covering all major clades, and probed the independence of evolution of (i) springboard trapping in *N. gracilis* and *N. pervillei* and (ii) the distinct component traits. Finally, we investigated the stochastic phenotypic variation of the lid angle and response to mechanical loading in a subset of 42 species for which a minimum of five replicates could be sampled (21, Table S2). While our data mainly reflect intraindividual

variation, we cannot exclude a minor genetic variability component (21). For lid surface waxes, we could not acquire enough samples to quantify intra-individual variation; however, scanning electron micrographs (Fig. S1) indicate that the presence, morphology and spatial distribution of lid wax crystals are highly variable between species.

Identical adaptations in two species originate from convergence

The orientation of the pitcher lid –recorded as deviation from horizontal– (Fig. 1A, G) is near-horizontal in both *N. gracilis* ($8.0 \pm 1.2^\circ$ [mean \pm s.e.m.]; $n = 68$) and *N. pervillei* ($8.8 \pm 1.8^\circ$; $n = 37$). These lid angles are significantly lower than the average lid angles of all 55 investigated *Nepenthes* species ($24.8 \pm 5.0^\circ$, lower 95% confidence interval = 15.0°). Thus, both species fulfil the first requirement for springboard trapping.

In both species, the lid's lower surface is covered by epicuticular wax 'pillars' (Fig. 1B, H) that differ drastically from the much more slippery wax platelets inside the pitcher (Fig. 1C, I, 22). Wax crystal morphology is determined by chemical composition (23). Gas chromatography-mass spectrometry of cuticular waxes (21) revealed similar chemical profiles for corresponding surfaces of *N. gracilis* and *N. pervillei* (Fisher-Freeman-Halton's exact test, d.f. = 5, $\chi^2_{\text{lid}} = 7.3$, $p_{\text{Bonferroni}} = 0.8$; $\chi^2_{\text{inner wall}} = 2.9$, $p_{\text{Bonferroni}} = 1.0$), but strong differences between the lid and pitcher wall in both species ($\chi^2_{\text{gracilis}} = 42.1$, $p_{\text{Bonferroni}} < 0.001$; $\chi^2_{\text{pervillei}} = 46.8$, $p_{\text{Bonferroni}} < 0.001$; Fig. 2). Lid waxes of both species were dominated by *n*-alcohols whilst the pitcher wall waxes consisted mainly of aldehydes, as in other *Nepenthes* species (24). Cuticular wax biosynthesis follows a stepwise pathway where C_2 building blocks are added to elongate fatty acid precursors and form very long-chain aliphatic compounds that can then be enzymatically modified into alcohols, esters, aldehydes, alkanes and other wax compounds (25). Due to this modular assembly process, shifts between major compound classes can be achieved relatively easily by redirecting substrate flux through different enzymatic pathways. Both species thus fulfil the second requirement for springboard trapping.

High-speed video analysis (21) confirmed that lids of both species respond to a drop impact with fast pivoting oscillations (Fig. 1E, K). *N. gracilis* lids had lower area and mass ($305 \pm 27 \text{ mm}^2$; $85 \pm 11 \text{ mg}$; $n = 11$) and higher resonant frequencies ($88 \pm 8 \text{ s}^{-1}$) than lids of *N. pervillei* ($836 \pm 102 \text{ mm}^2$; $318 \pm 48 \text{ mg}$; $58 \pm 5 \text{ s}^{-1}$; $n = 5$). For direct kinematic comparison using laser Doppler vibrometry, we thus selected relatively large *N. gracilis* lids ($315 \pm 46 \text{ mm}^2$, $87 \pm 13 \text{ mg}$, $92 \pm 5 \text{ s}^{-1}$; $n = 3$) and small *N. pervillei* lids ($252 \pm 39 \text{ mm}^2$; $80 \pm 9 \text{ mg}$, $81 \pm 18 \text{ s}^{-1}$; $n = 4$). When comparing similar lid masses and lever lengths, both species behaved almost identically. The initial down-stroke reached higher acceleration and speed and ended with a more abrupt deceleration than the subsequent, damped oscillations (Fig. 1F, L). Thus, lids of both species act as torsion springs generating high jerk forces (20), thereby fulfilling the third requirement for springboard trapping.

The striking similarity of springboard trapping in both species suggests that it could have originated either through evolutionary contingency, sharing a single origin (27), or independently, through convergent evolution. Of the 55 investigated species, 12 had horizontal lids and eight had pivoting lids, but only five species (including *N. gracilis* and *N. pervillei*) presented pillar-shaped crystals (Fig. 3). Moreover, *N. vieillardii* and *N. bicalcarata*, two species that stand in between *N. gracilis* and *N. pervillei* in the phylogeny (30) but were not included in

our analysis due to low sample sizes (21), lacked lid wax crystals (Fig. S2). We used a recent *Nepenthes* phylogenomic tree (30) to test if the composite trait and its components evolved independently in *N. gracilis* and *N. pervillei*. Ancestral state estimations (Fig. S3) and phylogenetic analyses (Figs. S4-S7, Tables S3, S4) strongly support an independent origin of springboard trapping and all of its three component traits in *N. gracilis* and *N. pervillei*.

Two alternative scenarios for the evolution of springboard trapping

The convergent origin of springboard trapping provides an opportunity to probe two alternative scenarios for the evolution of this composite trait: 1) a ‘directional selection’ scenario wherein all component traits evolve jointly and are under directional selection towards the state required for the synergistic combination; and 2) a ‘spontaneous coincidence’ scenario where the synergistic constellation occurs by chance, and the composite trait is subsequently fixed by selection for the novel function. The pool of possible random character state combinations that can occur is maximized when the phenotypic variation of each component trait is high and each component evolves independently.

A ‘directional selection’ scenario implies that (i) the components of the composite trait show correlated evolution and (ii) phenotypic variation is reduced when a component trait approaches the required state for springboard trapping, irrespective of the other traits. By contrast, a ‘spontaneous coincidence’ scenario posits that (i) each component evolved independently, and (ii) phenotypic variation in absence of the composite trait is higher than for the species with springboard trapping, irrespective of the trait value, as the individual traits are not under (strong) selection unless they occur in the beneficial combination. The two scenarios are not mutually exclusive: under a ‘mixed scenario’, some traits are under selection leading to exaptation (29) for the composite trait while high phenotypic variation in traits not under selection increases the odds for a synergistic coincidence.

Lack of correlated evolution between the three component traits supports the spontaneous coincidence scenario of composite trait evolution

To test for correlated versus independent evolution of some or all component traits we used two approaches. In both cases, we first excluded marginal cases (near-horizontal lid orientation, pivot-like loading response, inconsistent wax crystal presence or shape) when coding trait presence, and then repeated the analysis including them. We used the Bayesian implementation of Pagel’s model of correlated evolution in corHMM (21), a modelling environment that allows to test for correlation among all three traits at once. We independently confirmed our results by testing for correlation in pairwise combinations of traits using BayesTraits V3 (21).

Altogether, we fitted nine models of trait evolution that ranged from traits evolving independently to a model where the evolution of each of the three traits is linked to the other two traits (21). To account for a possible mixed scenario, we included models where two of the three traits evolved in a correlated fashion while the third evolved independently (Fig. 4). Each of these models represents a different hypothesis about how these traits evolved, and by comparing the fit of each model to the observed trait distribution we were able to select the hypothesis most supported by our data. The fully correlated models were consistently outperformed (Fig. 4,

Tables S3, S4), thus strongly favoring a scenario of independent evolution of the component traits and supporting the ‘spontaneous coincidence’ hypothesis for the evolution of springboard trapping.

5 **Stochastic variation of lid angles supports the spontaneous coincidence scenario**

Next, we measured lid angles of 900 pitchers from 42 *Nepenthes* species to test whether the stochastic (intra-individual) phenotypic variation was generally reduced in species with predominantly horizontal lids (indicating directional selection), or in springboard-trapping species only (indicating spontaneous coincidence). Horizontal lids might offer more effective rain protection, although a larger lid could compensate for a steeper angle (26). Horizontal lids might also be advantageous for photosynthesis; however, pitchers have been shown to contribute little to photosynthesis (59), since lids are very small compared to the leaf lamina, and a broad range of lid angles may be equally effective for light capture (60). If there was strong selection for a horizontal lid, we would expect the median of lid angles to be close to 0°. For our 55 species, however, median lid angles (23°) were clearly above horizontal. More importantly, there was no significant difference in the standard deviation (s.d.) of lid angles between species with near-horizontal lids ($-20^\circ \leq \text{lid angle} \leq 20^\circ$, median s.d. = 16.14) and other species (median s.d. = 18.16; Mann-Whitney U test, $n_1 = 20$, $n_2 = 22$, $U = 186.0$, $p = 0.40$). Lid angle variation was also not correlated with mean lid angle (Spearman test, $n = 42$, $\rho = 0.10$, $p = 0.53$).

Deviation from horizontal lid angles is detrimental for the springboard function of the lid. Therefore, we expect stabilizing selection to limit the variability of lid angles in springboard-trapping species, regardless of the evolutionary scenario. As expected, the lid angles of *N. gracilis* (standard deviation s.d. = $\pm 9.9^\circ$, $n = 68$) and *N. pervillei* (s.d. = 11.0° , $n = 37$) showed lower than average stochastic variation (in the bottom 20% of values; mean s.d. for 42 species = 17.4° ; Figs. 3, S8). Altogether, our data suggest that the lid angle is not under directional selection and its evolution follows the ‘spontaneous coincidence’ scenario.

30 **Stochastic variation of lid loading responses points to spontaneous coincidence or mixed scenario**

To assess variation in lid loading response, we manually loaded lids and assigned one of six deformation types (pivoting, pivot-like, bending, buckling, mixed bending-buckling, and other, more complex responses; Fig. 5; 21). *N. gracilis* and *N. pervillei* exhibited close to 100% pivoting – the crucial response for springboard trapping (index of dispersion I_D for *N. gracilis* = 69, $\chi^2 = 5181.3$, d.f. = 75, $p < 0.01$; and *N. pervillei* $I_D = 19$, $\chi^2 = 447.9$, d.f. = 24, $p < 0.01$; these were the highest and fourth-highest I_D of all species, respectively; $I_D \gg 1$ indicates highly uniform response). Other species typically showed a range of loading responses (median $I_D = 3$, 95% confidence interval = 35.1; Figs. 3, S9). Thus, the loading response was less variable in springboard-trapping species than in most other species, as expected. However, lid loading response variability was also reduced in two out of six other species with a pivoting lid response (*N. tobaica*, $I_D = 35$, and *N. mikei*, $I_D = 34$; Kruskal-Wallis test, $H = 9.83$, d.f. = 2, $p = 0.008$; Fig. S9), indicating that this trait might be developmentally constrained or under selection for a different function.

Pivoting requires the lid to be mechanically reinforced to resist bending or buckling (20), thus implying increased construction costs. Indeed, *N. gracilis* pitchers have relatively high lignin content compared to other sympatric *Nepenthes* species (31). Structural compounds such as cellulose and lignin are comparably ‘cheap’ for carnivorous plants which are generally limited by mineral nutrients, not by water and light (63). Tissue reinforcement is also correlated with trap longevity (31). Stiffer lids and their pivoting load response might thus result from selection for more durable traps, with certain life histories potentially favoring springboard trapping.

Lid loading response could also be under selection for damage prevention (61), and for effective water shedding (62). For both functions, we would expect any elastic deformation (pivoting, pivot-like, bending, twisting) to be effective, and all loading responses except buckling to be favourable. This is corroborated by the distribution of predominant loading responses across the 42 investigated species, where all responses except buckling (two species) and bending-buckling (none) are common (Fig. S9). The most common response is ‘variable’ (no predominant response, 16 species), which further points to the absence of significant selective pressure for a specific load response. Although pivoting as a predominant lid loading response was only found in eight out of 42 investigated species, and intra-individual variation of lid loading response was high in 38 out of 42 species, we cannot completely rule out that there might be a degree of selection for pivoting lids.

Conclusions

Our study reveals convergence of a composite trait in tropical pitcher plants which likely arose by ‘spontaneous coincidence’ of the component traits, possibly in a mixed scenario combining directional selection in one component with high plasticity *sensu lato* (14) in another. Trait plasticity, whether environmentally induced or stochastic in nature, enhances phenotypic diversity and can become canalized when chance combinations entail a synergistic function. Accordingly, Levis *et al.* (32) demonstrated the emergence of a carnivorous tadpole morph with three independent morphological adaptations in Scaphiopodidae species, arising from high phenotypic plasticity and subsequent adaptive refinement of the new composite trait. Our findings highlight the importance of stochastic phenotypic variation, not only for selection to act on, but also for increasing the likelihood of co-option into a new function (29). Importantly, this ‘spontaneous coincidence’ mechanism for the evolution of composite traits is applicable to any type of variation on which selection can act when beneficial combinations arise by chance. Our findings emphasize the role of stochasticity in composite trait evolution and call for further investigation into the role of plasticity, and stochastic phenotypic variation in particular.

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Supplementary Materials:

Materials and Methods

Figures S1-S9

Tables S1-S4

Movies S1-S2

5 **Figure captions:**

10 **Fig. 1.** Adaptations for springboard trapping in *Nepenthes gracilis* (A-F) and *N. pervillei* (G-L). (A) and (G) Pitchers with near-horizontal lid orientation. (B) and (H) Lid wax ‘pillars’ provide limited grip for insects. (C) and (I) Wax platelets render the internal pitcher surface extremely slippery. (D) and (J) A drop impact dislodges an ant from the lid. (E) and (K) High-speed video tracking and (F) and (L) laser Doppler vibrometry of three *N. gracilis* (p1-3) and four *N. pervillei* (p4-7) lids show that all lids respond with a rapid initial downstroke, followed by regular damped oscillations.

20 **Fig. 2.** Cuticular wax composition (mean \pm s.d.) on the lower lid surface (upper part of the graph) and pitcher inside (lower part of the graph) of *N. gracilis* (white bars) and *N. pervillei* (grey bars). Both species show similar differences between the lower lid surface (predominantly alcohols) and the pitcher inside (predominantly aldehydes). Numbers indicate C-chain lengths. Bs = β -sitosterol, Aa = α -amyrin, Ba = β -amyrin, Fr = Friedelin. For statistics see main text.

25 **Fig. 3.** Mapping the presence of the three component traits of springboard trapping on a recently published *Nepenthes* phylogeny (30) reveals their independent evolution. Symbols indicate trait presence; lighter colors denote marginal cases (near-horizontal lid angles, pivoting-like responses, and weak/inconsistent lid wax) (21). For detailed analysis of trait evolution see main text, Fig. 4, and Tables S3 and S4. Trait variability data reveal lower stochastic phenotypic variation of lid angles and lid loading responses in springboard-trapping *N. gracilis* and *N. pervillei* than in species without this composite trait. For detailed analysis of trait variability see main text and supplementary Figures S8 and S9.

35 **Fig. 4.** Testing for the independent, mixed or correlated evolution of the springboard trapping component traits. Evolutionary models compared to test the predictions for component trait evolution under directional selection, spontaneous coincidence, and under a mixed scenario, all implemented in corHMM (21). Illustrations show the evolutionary transition from wax crystal absence (top row of trait cases) to presence (bottom row), and three exemplary model cases: fully independent (left), wax crystals and load response correlated (middle), and all three traits

correlated (right). The proportions of 1,000 simulations supporting each scenario are reported below for simulations with marginal cases (light colored symbols) for each trait either coded as present or absent. The simulations strongly support the independent evolution of all three component traits, and therefore the spontaneous coincidence scenario of composite trait evolution. (For detailed model descriptions and results see 21 and Table S4).

Fig. 5. Lid angles (A-E), loading responses (F-J), and surface waxes (K-O) in *Nepenthes*. (A) Horizontal, *N. pervillei*. (B) Steeply angled, *N. albomarginata*. (C) Steeply angled, *N. inermis*. (D) Upright, *N. biak*. (E) Reflexed, *N. ampullaria*. (F) Pivoting, *N. gracilis*. (G) Pivot-like, *N. adnata*. (H) Bending, *N. andamana*. (I) Buckling, *N. burbridgeae*. (J) Mixed bending-buckling, *N. vogelii*. (K) Wax ‘pillars’, *N. pervillei*. (L) Wax ‘pillars’, *N. khasiana*. (M) Wax spikes, *N. mira*. (N) Scarce wax platelets, *N. dactylifera*. (O) Smooth wax film, *N. glabrata*. Scale bars = 2µm.

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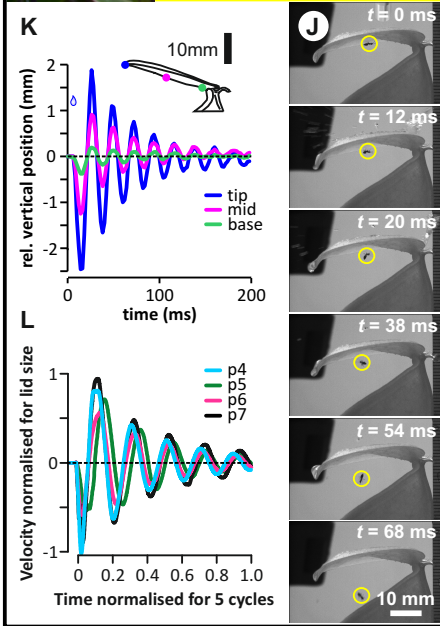
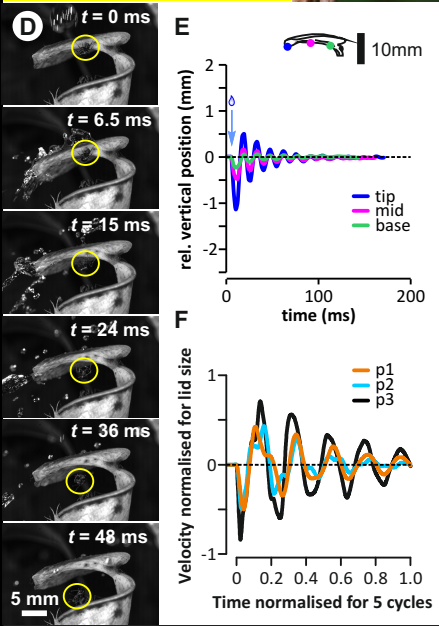
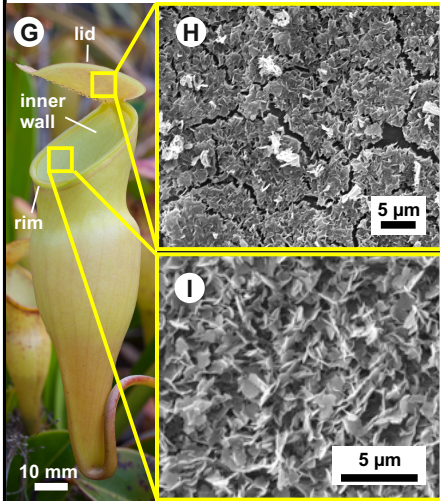
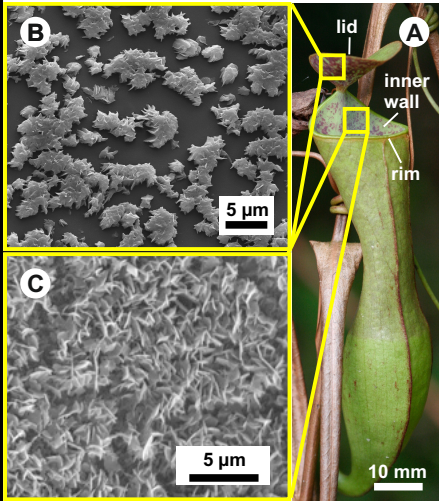
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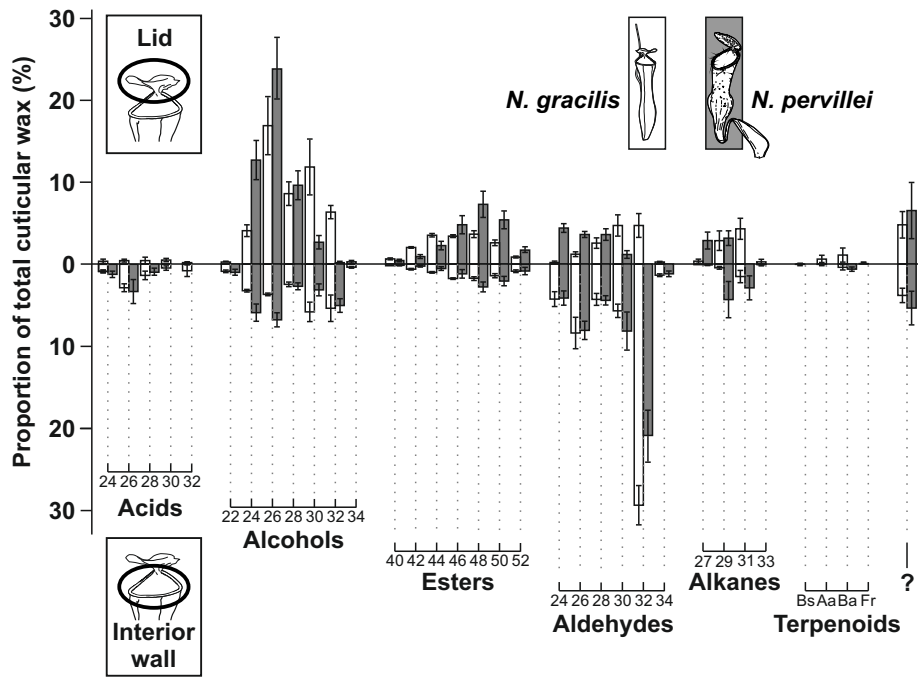
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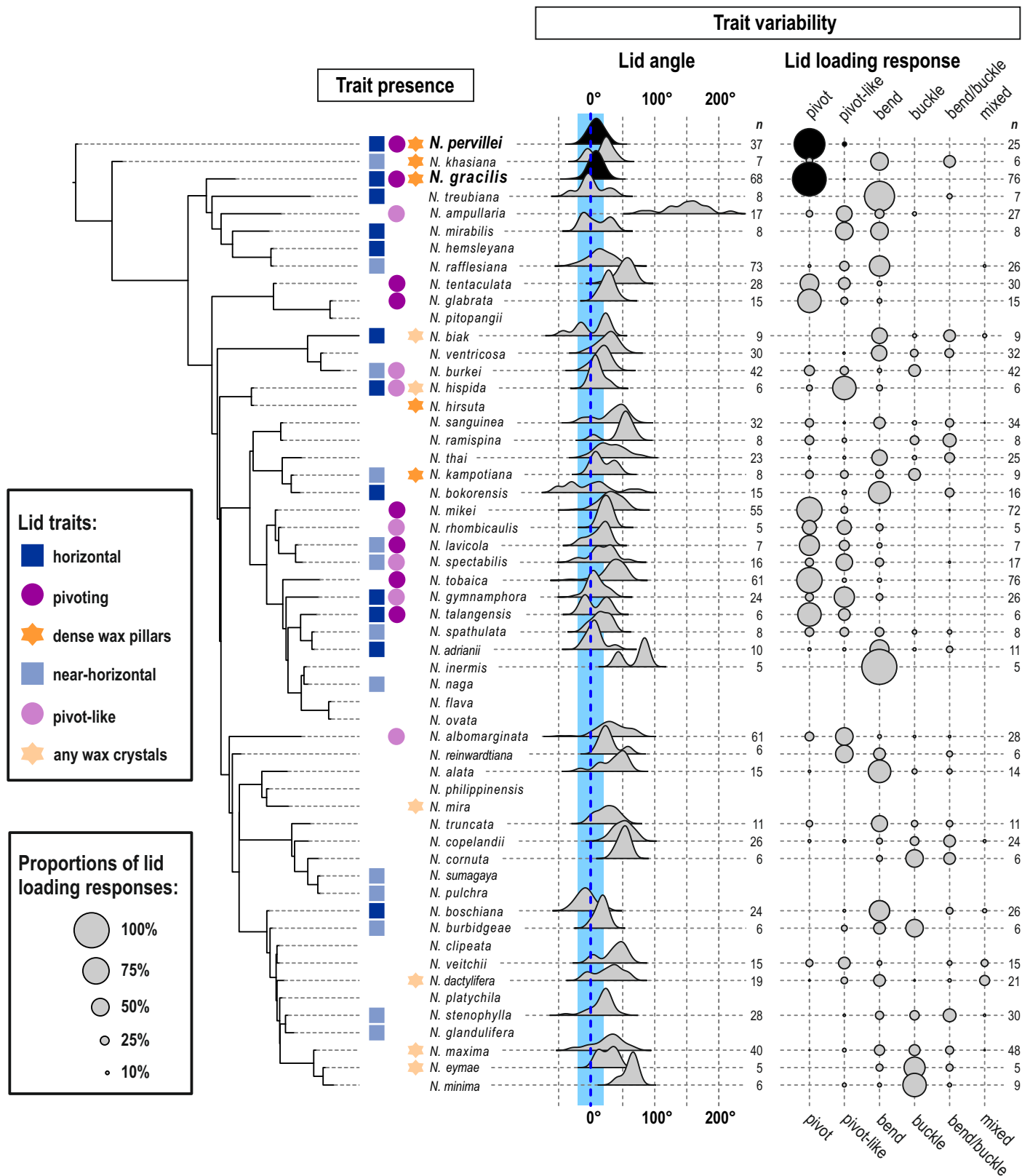
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N. gracilis

N. pervillei







Evolutionary scenarios:

Spontaneous
Coincidence

Mixed

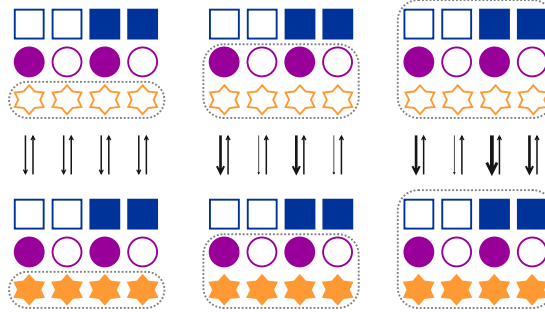
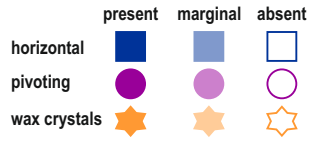
Directional
Selection

Component trait evolution:

Independent

Correlated

Lid traits:



Supported simulations:

Cases included as trait presence

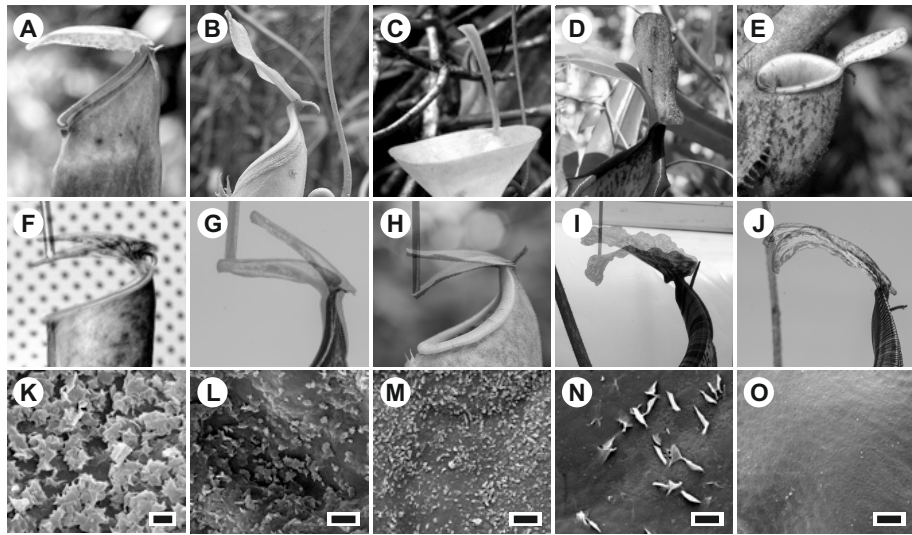


All independent

2 traits correlated

All correlated

	100%	0%	0%
	100%	0%	0%
	100%	0%	0%
	100%	0%	0%
	100%	0%	0%
	88.1%	11.9%	0%
	100%	0%	0%
	79.8%	20.2%	0%





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