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Écologie, Développement et Évolution des plantes carnivores à urnes du genre *Nepenthes*

par

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À Jean-Jacques Labat,
pour son amitié et son humanisme ;

À mes grands-parents,
dont l'amour plein a bercé mes jeunes journées.

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INTRODUCTION GÉNÉRALE

Je n'ai encore rencontré personne qui n'ait entendu parler des plantes carnivores et n'en ait sa propre idée. Si la fascination brutale qu'elles suscitent n'a pas fléchi, de leur découverte à nos jours, c’est probablement dû à la réunion du statut de curiosité botanique et de traits considérés comme exclusivement animaux : la capacité d'attraper et digérer des proies avec des organes morphologiquement atypiques, souvent mobiles. Darwin est le premier à s’intéresser à la fois au mouvement chez les plantes (Darwin, 1865, Darwin, 1882, Whippo & Hangarter, 2009) et à démontrer la réalité de la carnivorie végétale (Darwin, 1875). Sa correspondance atteste qu'il fit des plantes carnivores un défi personnel : "*I care more about* Drosera *than the origin of all the species in the world*" (Darwin, 1860). Darwin, a transformé les plantes carnivores d’anecdotes végétales en modèle de choix pour les botanistes, les physiologistes et les écologues de l'évolution.

Le manuscrit qui suit est focalisé sur le genre de plantes carnivores à urnes *Nepenthes* et vise à travers plusieurs approches intégratives à amener plusieurs éléments de connaissance nouveaux sur leur évolution, écologie et développement, notamment en ce qui concerne leurs systèmes de piégeage. Cette thèse débute par une introduction générale qui passe en revue l’état des connaissances actuelles sur les plantes carnivores et vise à montrer qu’elles sont un modèle de choix en écologie évolutive. Elle se focalise ensuite sur le genre *Nepenthes* et les questions de recherche explorées durant cette thèse.

LES PLANTES CARNIVORES : UN MODÈLE DE CHOIX EN ÉCOLOGIE ÉVOLUTIVE

Le syndrome carnivore

Mycohétérotrophie mise à part, la plupart des plantes synthétisent leurs hydrates de carbone à partir du dioxyde de carbone atmosphérique, réduit grâce à l'énergie lumineuse interceptée au niveau de leur système photosynthétique (Larcher, 2003). Elles obtiennent d'autre part l'eau et les éléments minéraux essentiels à leurs métabolismes à partir de leurs systèmes racinaires, directement ou par l'intermédiaire de symbioses mycorhyziennes (Selosse *et al.*, 2007, Selosse & Roy, 2009). La disponibilité des nutriments essentiels (N, P, K et leurs ratios relatifs) sont les principaux facteurs limitants de la croissance des plantes après l'eau et la lumière (Chapin III, 1980, Aerts & Chapin III, 2000). Les environnements où les nutriments sont rares ou indisponibles sont propices à l'évolution de systèmes de nutrition alternatifs, où certains éléments essentiels sont directement dérivés d'arthropodes (Figure 1). Que ce soit à travers un mutualisme ou une relation prédateur-proie, les relations trophiques entre plantes et animaux sont alors partiellement inversées (Thompson, 1981). Les plantes myrmécotrophes tirent une partie de leur nourriture des débris et des fèces d'une fourmi mutualiste et les plantes carnivores capturent des arthropodes dans leurs environnement (Benzing, 1986, Beattie, 1989).

Une plante carnivore présente des adaptations structurelles et physico-chimiques qui concourent à l'attraction, la capture et la digestion d'arthropodes dont l'assimilation des éléments dérivés complète son alimentation minérale (Juniper *et al.*, 1989). Les plantes carnivores sont capables, d'acquérir des nutriments non seulement par leurs appareils racinaires, souvent réduits mais fonctionnels, mais aussi par les feuilles qui gardent par ailleurs des capacités photosynthétiques (Lüttge, 1983, Adamec, 1997, Adamec, 2005). Ces adaptations sont regroupées sous le nom de *syndrome carnivor*e, terme dont l'utilité est double.

Le « syndrome carnivore » fournit tout d'abord un critère opérationnel (et accepté) pour statuer sur la carnivorie des espèces. Si la conjonction de l'attraction, de la capture et de la digestion d'arthropodes définit une plante carnivore, cette définition exclut donc de fait les feuilles exclusivement collantes considérées comme une protection anti-herbivorie (capture uniquement), les nectaires floraux et autres adaptations liées à l'entomogamie (attraction uniquement), et enfin la production d'enzymes protéolytiques lors de la germination et la dissolution des albumens (digestion uniquement). C'est donc la combinaison de traits physiologiques, largement présents indépendamment chez les végétaux, qui confère le statut de plante carnivore.

Par ailleurs, la discrétisation du syndrome carnivore en composantes fonctionnelles est utile au-delà du diagnostic carnivore : elle permet de comparer les homologies fonctionnelles impliquées dans la carnivorie. Replacée dans une perspective évolutive, la comparaison des structures anatomiques et morphologiques permet de distinguer les convergences évolutives des analogies (Albert *et al.*, 1992, Cameron *et al.*, 2002). Les pièges à urnes de *Nepenthes* (Caryophyllales) et de *Cephalotus* (Ericales) se ressemblent mais les carnivories ont évolué indépendamment et la ressemblance n'est qu'une pure convergence morphologique et fonctionnelle. À l'inverse, les genres *Nepenthes*, *Drosera*, *Drosophyllum* sont frères même si le fonctionnement de leurs pièges est totalement différent (Figure 2).

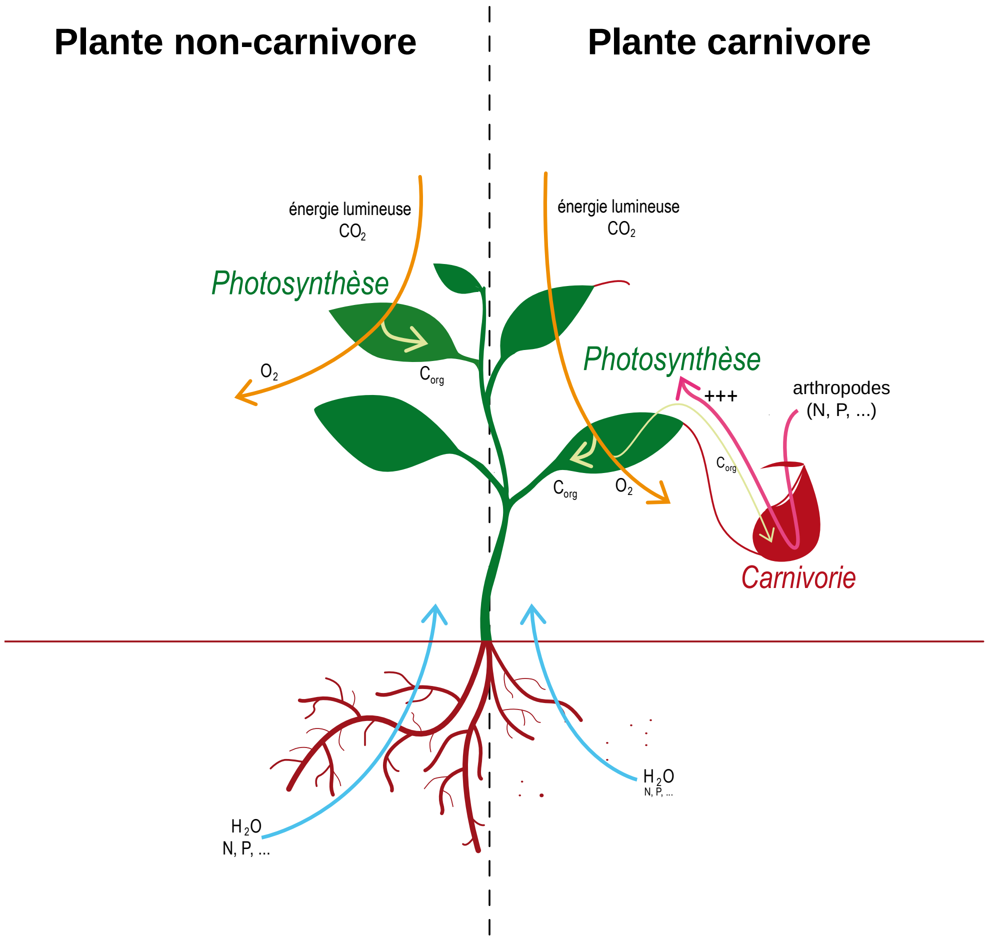
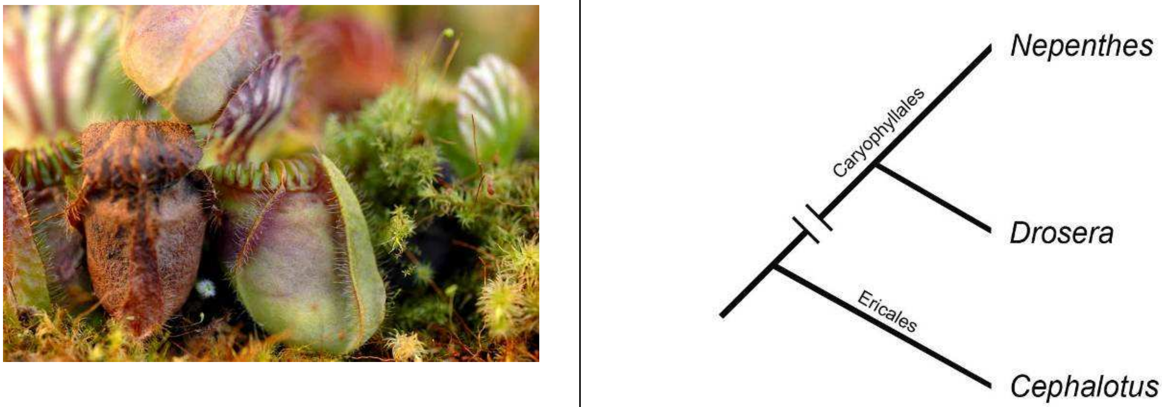


Figure 1: Physiologie d'une plante carnivore. Toute comme une plante non carnivore, une plante carnivore métabolise en carbone organique, lors de la photosynthèse, le dioxyde de carbone atmosphérique avec les éléments puisés par son appareil racinaire. Une partie de ce carbone organique est investi dans une structure moins photosynthétique qu'un limbe foliaire, le piège, mais dont la capacité à dériver des éléments essentiels tels que l'azote et le phosphore via la capture et la digestion d'arthropodes augmente en retour le potentiel photosynthétique de la plante.

Nepenthes ampullaria

Drosera rotundifolia



Cephalotus follicularis

Figure 2 : Les plantes carnivores, un bon laboratoire de l'évolution morphologique et fonctionnelle. De gauche à droite et de bas en haut : i) quelques urnes de *Nepenthes ampullaria*, ii) une rosette *de Drosera rotundifolia*, iii) quelques urnes de *Cephalotus follicularis*, iv) relations phylogénétiques des trois genres présentés. Malgré la forte ressemblance morphologique entre les pièges des genres *Nepenthes* (Caryophyllales) et ceux des *Cephalotus* (Ericales), ces deux formes ont évolué indépendamment alors que le pièges des *Nepenthes* dérivent de feuilles à mucilage comparable à celles des *Drosera* actuels.

Écologie évolutive des plantes carnivores

Écologie des plantes carnivores

Les préférences écologiques des plantes carnivores sont assez similaires. Parmi les plus caractéristiques, on peut citer (Juniper *et al.*, 1989) :

- Un appareil racinaire faiblement développé, l'alimentation minérale racinaire étant partiellement remplacée par la carnivorie, majoritairement foliaire ;
- Une préférence pour les sols très pauvres et acides, saturés en eau, voire fréquemment inondés ;
- Une préférence pour les milieux très ensoleillés;
- Une faible résistance à la compétition dans les autres milieux, qui peut être vue comme une conséquence de la bonne capacité à coloniser les milieux pauvres (Levins & Culver, 1971, Yu & Wilson, 2001).

À l'échelle mondiale, ces habitats sont la plupart du temps fragmentés, perturbés ou détruits et la plupart des plantes carnivores sont en danger d'extinction (Cheek & Jebb, 2001, Gotelli & Ellison, 2002, Moody & Green, 2010). Toutes les espèces du genre *Nepenthes* figurent par exemple dans les appendices I (*Nepenthes rajah* et *N. khasiana*) et II (toutes les autres espèces du genre) de la CITES, qui recensent respectivement les espèces animales et végétales considérées comme en extinction ou en voie de l'être, et dont le commerce est interdit ou soumis à des règles draconiennes (CITES, 2010).

Un modèle coût-bénéfice de l'évolution de la carnivorie

Le syndrome carnivore implique un coût pour la plante : sécrétion de nectar, d'odeurs, mise en place et entretien de structures spécialisées dont l'efficacité photosynthétique est moins importante que celle d'un limbe classique (Ellison & Farnsworth, 2005, Osunkoya *et al.*, 2007, Pavlovic *et al.*, 2007, Farnsworth & Ellison, 2008, Karagatzides & Ellison, 2009). Par ailleurs, les concentrations des feuilles en azote et phosphore (et leur ratio) sont considérées comme limitantes (Ellison, 2006). Si les coûts sont clairs, quels bénéfices la carnivorie procure-t-elle ?

Givnish a proposé en 1984 un modèle d'évolution de la carnivorie qui repose sur une balance coûts/bénéfices pour des plantes en milieu pauvres en nutriments (Givnish *et al.*, 1984). Les grandes lignes de ce modèle n'ont pas été modifiées car sa simplicité le rend général pour toutes les plantes carnivores. L'idée en est la suivante : dans un milieu pauvre en nutriments, mais non limitant en ressources lumineuse et hydrique, les coûts carbonés liés à la carnivorie sont compensés par l'acquisition de nutriments-clés qui augmentent l'efficacité photosynthétique globale. Le modèle biologique qui a inspiré le modèle écophysiologique de Givnish est *Brocchinia reducta* (Bromeliaceae), qui affectionne les environnements ouverts et gorgés d'eau du nord de l'Amérique du Sud et satisfait donc les conditions du modèle. Benzing d'une part, puis Ellison & Gotelli (Benzing, 1987, Ellison *et al.*, 2003) proposent plutôt une vision plus ouverte de ce modèle, un espace le long des trois gradients liés par des *trade-offs* d'humidité, d'ensoleillement et de disponibilité des ressources, dans lequel un coût lié à la carnivorie peut être compensé et de là, la carnivorie sélectionnée puis spécialisée. Dans un environnement légèrement fermé, mais détrempé et très pauvre, la carnivorie peut être avantageuse.

Valeur adaptative de la carnivorie

La valeur adaptative de la carnivorie est clairement établie. La capture d'insectes favorise la croissance, le nombre de fleurs et la production de graines chez tous les genres étudiés (Thum, 1988, Schulze & Schulze, 1990, Schultze *et al.*, 1997, Zamora *et al.*, 1997, Thoren & Karlsson, 1998, Göran & Harms, 2003). Ces études reposent sur la comparaison entre plantes contrôles et plantes dont les pièges ont été isolés, soit au contraire fournis en insectes ou directement fertilisés. L'azote, le phosphore et leurs ratios respectifs sont en concentrations limitantes chez les espèces étudiées (Ellison & Farnsworth, 2005, Ellison, 2006, Pavlovic *et al.*, 2007). À l'aide de marqueurs isotopiques de l'azote on peut montrer qu'une proportion variant entre 20 % et 80 % de l'azote total des genres étudiés est dérivé des insectes (Schultze *et al.*, 1997, Schulze *et al.*, 2001, Millett *et al.*, 2003).

Un tour du monde des plantes carnivores

La carnivorie n'est pas le fait d'une lignée évolutive unique. Plus de 600 espèces (soit 0,5 % des Angiospermes) réparties en 18 genres, 11 familles et 5 ordres sont décrites à ce jour et la carnivorie est apparue *a minima* 6 fois au cours de la diversification des Angiospermes, au sein des Mono- et des Dicotylédones (McPherson, 2009, The Angiosperm Phylogeny Group, 2009). Les plantes carnivores sont un laboratoire de l'évolution : la variété de leurs systèmes de piégeage offre un modèle de choix pour comparer les adaptations qui concourent à attirer, capturer, digérer des insectes.

De plus, les plantes carnivores sont cosmopolites, elles croissent des régions tropicales aux régions arctiques, sont terrestres, semi-aquatiques (par ex. *Utricularia*) voire entièrement aquatiques (par ex. *Aldrovanda*). La plupart d'entre elles croissent dans des environnements ouverts, sur des sols acides et/ou très drainants.

On distingue plusieurs types fonctionnels de piégeage, qu'ils aient ou non une origine évolutive commune : pièges à mâchoires des *Dioneae* (Caryophyllales) et *Aldrovanda* (Caryophyllales) ; pièges à succion des *Utricularia* (Lamiales); pièges à feuilles jointives de *Brocchinia reducta* (Poales), pièges à feuilles collantes des *Drosera* (Caryophyllales), *Pinguicula* (Lamiales); pièges à urnes des, *Heliamphora* (Ericales), *Sarracenia* (Ericales), *Darlingtonia* (Ericales), *Cephalotus* (Oxalidales), et *Nepenthes* (Caryophyllales – Figure 3).

Peu de matériel fossile est disponible pour les plantes carnivores. Mis à part quelques pollens et graines, seule *Archaeamphora longicervia*, du Crétacé inférieur (100 – 140 Ma) et morphologiquement semblable aux *Sarracenia*, est décrite avec certitude (Li, 2005). A l'heure actuelle, la paléobotanique ne peut nous éclairer sur l'évolution des plantes carnivores et nous devons composer avec leur diversité actuelle.

LES PLANTES CARNIVORES À URNES DU GENRE *NEPENTHES*

Une courte histoire du genre

En 1658, Etienne de Flacourt, directeur général de la compagnie française de l'orient et gouverneur de Madagascar, décrit *Anramatico*[1] – nous sommes dans le système prélinnéen –, qui deviendra plus tard *Nepenthes madagascariensis*, une des deux espèces endémiques de l'île, à l'extrémité occidentale de l'aire de répartition du genre. Linné introduit le siècle suivant la nomenclature binomiale et réunit dans le genre *Nepenthes*, *Nepenthes madagascariensis* et *Nepenthes distillatoria*, découverte entre temps au Sri Lanka, dont il fait l'espèce type du genre. Le nom de genre est choisi par Linné en référence à la drogue Nepenthe utilisée par Hélène dans l'*Odyssée* d'Homère[2] : « si ce n'est celle d'Hélène, ce sera celle de tous les botanistes »

S'ensuit un intérêt croissant pour les espèces du genre, dont on décrit plusieurs espèces à Bornéo et Sumatra, points chauds d'endémisme et de diversification du genre, et qui sont ramenées en Europe et mises en culture. Au moment où, en Europe, les muséums d'histoire naturelle supplantent peu à peu les cabinets des curiosités privés, elles deviennent tout à la fois des pièces de collection horticoles et des sujets d'étude à part entière.

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[1] « L'Anramatico est une plante qui croît de la hauteur de deux coudées [env. 90cm] […] dont les fleurs sont au bout des feuilles comme des chopinettes, un petit vase, qui a son couvercle, et qui ne se lasse pas de se remplir d'eau lorsqu'il pleut ».

[2] « Et alors Hélène, fille de Zeus, eut une autre pensée, et, aussitôt, elle versa dans le vin qu'ils buvaient un baume, le Nepenthe, qui donne l'oubli des maux. Celui qui aurait bu ce mélange ne pourrait plus répandre des larmes de tout un jour, même si sa mère et son père étaient morts, même si on tuait devant lui par l'airain son frère ou son fils bien-aimé, et s'il le voyait de ses yeux ».

Figure 4 : Un tour d'horizon de 16 genres carnivores. De haut en bas et de gauche à droite. *Sarracenia* et le genre monotypique *Darlingtonia*, les plantes à urnes nord-américaines. *Heliamphora*, genre de plantes à urnes sud-américaines, affectionant notamment les tepuis amazoniens. *Nepenthes*, le genre asiatique de plantes carnivores à urnes. *Drosera*, genre cosmopolite et *Drosophyllum* genre monotypique et leurs pièges à mucilage, sont les genres frères de *Nepenthes*. *Aldrovanda* et *Dioneae*, genres apparentés, l'un aquatique, l'autre terrestre et produisant des pièges à mâchoires. *Byblis*, genre de plante carnivore principalement nord-australien et qui produit des feuilles à mucilage. *Cephalotus*, genre de plante à urnes monotypique également australien. *Triphyophyllum peltatum*, la seule espèce des Dioncophyllaceae, au sein du genre monotypique à être carnivore. *Genlisea* qui produit des pièges tubulaires et en forme de tire-bouchon au sein de Lentibulariaceae, qui regroupent également *Pinguicula* qui produit des pièges à glu. *Utricularia*, le genre de plantes carnivores le plus riche en espèce, dont on distingue les pièges à succion. *Brocchinia reducta* et *Catopsis berteroniana* dont les feuilles engainantes forment des reservoirs d'eau, où les insectes sont piégés. On distingue la cire épicuticulaire poudreuse de *Brocchinia reducta*. Photos Wikimedia Commons, Vincent Bonhomme.

Danser publie en 1928, un ouvrage intégralement consacré au genre *Nepenthes* qui sera le premier à proposer une classification infragénérique et des éléments de biogéographie, reconnaissant alors 51 espèces dont les aires de répartition commençaient à être connues (Danser, 1928). Le milieu du XX^{e} siècle voit la multiplication des recherches sur les plantes carnivores et la publication d'ouvrages de référence tels que celui de Lloyd en 1942, et celui de Juniper qui, accompagné de Robins et Joël, publie en 1989 une synthèse considérée aujourd'hui encore comme la référence de la communauté (Lloyd, 1942, Juniper *et al.*, 1989). Clarke publie pour le genre *Nepenthes* deux ouvrages de référence sur ses voyages et recherches en 1997 et 2001 (Clarke, 1997, Clarke, 2001). Cheek et Jebb fournissent, les mêmes années, une révision taxinomique complète et documentée du genre reconnaissant alors 87 espèces (Jebb & Cheek, 1997, Cheek & Jebb, 2001). Parallèlement, les nouvelles descriptions se multiplient avec les voyages d'exploration et les dernières publications font état de 120 espèces (McPherson, 2009).

Description du genre

Feuilles et urnes

Les limbes foliaires sont lancéolés et leurs pétioles sont légèrement engainants sur l'axe. Leurs pièges, appelés urnes (*pitchers* [en], *sumboi* [bahasa malayu, bahasa indonesia]) sont des feuilles modifiées. Les modalités morphogénétiques du piège sont débattues mais les urnes sont soit issues de l'enroulement et de la fusion des marges du limbe (MacFarlane, 1889 in Juniper *et al.* 1989, McPherson 2009) ou de l'expansion de la nervure centrale (Troll, 1932 in Juniper *et al.* 1989, McPherson 2009). Les urnes sont portées par une vrille qui les relie au limbe foliaire (Figure 4).

Inflorescence, dispersion et germination

Les inflorescences sont majoritairement des grappes (quelques exceptions d'inflorescences en panicules) et le genre *Nepenthes* est dioïque. La biologie florale est, à quelques exceptions près (Kaul, 1982, Kato, 1993, Moran, 1993, Adam, 1998, Chua, 2000), peu étudiée sur le terrain mais les espèces du genre sont probablement entomogames (Cheek & Jebb, 2001). Les inflorescences qui peuvent porter plusieurs centaines de fleurs odorifères et nectarifères attirent une variété d'insectes volants et de fourmis.

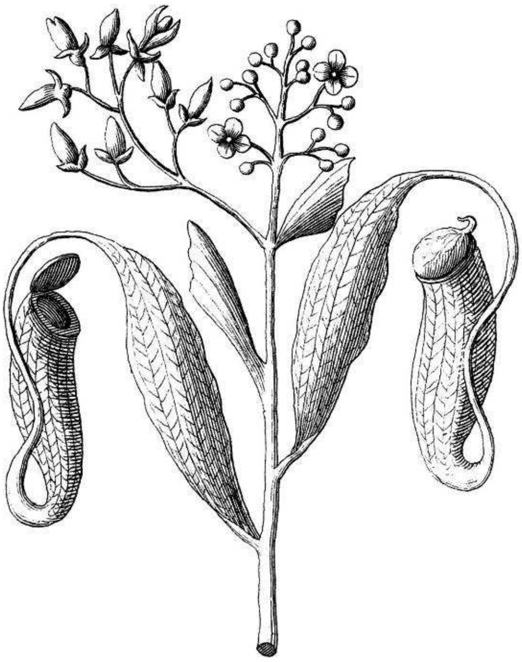

Figure 5: Organisation générale d’un axe de *Nepenthes*. Les urnes sont des feuilles modifiées, reliées au limbe par une vrille. Les inflorescences sont des grappes. La gravure correspond à la deuxième espèce décrite *Nepenthes distillatoria*. Gravure Plukenet's 1696 *Almagestum*).

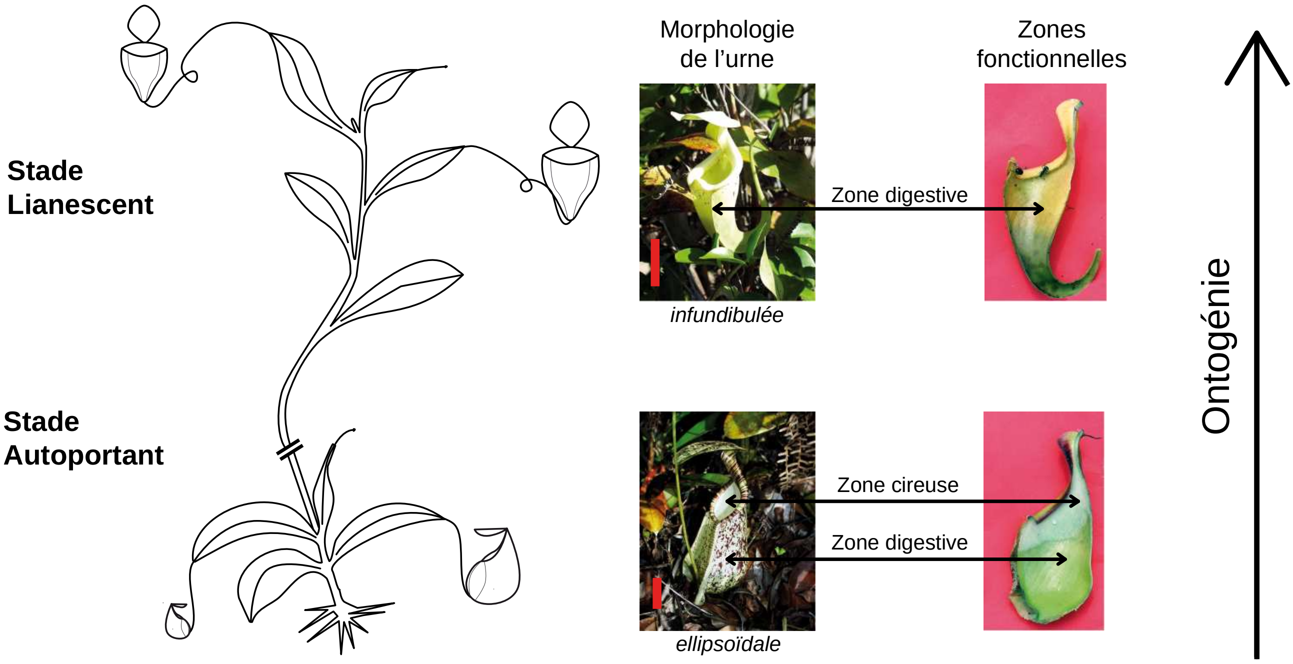

Figure 6: Transitions architecturale, morphologique et fonctionnelle de *Nepenthes rafflesiana* var. *typica*.

Les graines sont quasiment exalbuminées et très effilées. La dispersion est selon toute vraisemblance exclusivement barochore : la faible masse des graines, produites par des infrutescences le plus souvent situées à plusieurs mètres du sol, permet une dispersion à courte distance. La germination se déroule de quelques semaines à quelques mois après le semis. Dès les premières feuilles post-cotylédonnaires, des urnes minuscules (quelques mm) et semble-t-il fonctionnelles sont produites (*obs. pers.*).

Changements morphologiques au cours de l'ontogénèse

Le cycle de vie des plantes est constitué d'une série de changements développementaux de la germination à l'acquisition de la sexualité, comme par exemple l'allongement des entre-nœuds ou la taille et la forme des feuilles. Ces changements sont généralement minimes et progressifs au cours de l'ontogénie de la plante et ce type de développement est dit homoblastique (Putz & Mooney, 1991a).

En revanche, les plantes qui expérimentent des contraintes très différentes au cours de leurs vie, comme par exemple les plantes aquatiques selon qu'elles soient totalement immergées ou non (Bradshaw, 1965, Wells & Pigliucci, 2000, Minorsky, 2003) présentent des changements plus drastiques. Les lianes présentent par exemple une plasticité phénotypique importante (Rowe & Speck, 2005). Ce sont des plantes ligneuses connaissant, au cours de leur ontogénie, une transition plus ou moins rapide d'un faisceau de traits comme la taille, la forme et l'anatomie des feuilles, la phyllotaxie, la capacité de régénération, la réponse tropique, l'acquisition de la sexualité, etc. Leur développement est alors qualifié d'hétéroblastique (Lee & Richards, 1991). Les lianes perdent par exemple leur autoportance et s'accrochent à un support à l'aide d'organes spécialisés comme les vrilles, crochets, ventouses, etc., ce qui leur permettent de réallouer une partie de ses ressources à la croissance végétative ou la sexualité.

Le genre *Nepenthes* est lianescent, et produit les individus parmi les plus imposants chez les plantes carnivores, pouvant dépasser les 15 mètres de haut. Le genre *Nepenthes* a un développement hétéroblastique et on voit, selon les espèces, des modifications plus ou moins importantes de ses organes, et notamment de ses urnes au cours du développement. On distingue les urnes du bas ou terrestres (*lower pitcher*s) des urnes du haut ou aériennes (*upper pitchers*). Les urnes terrestres reposent sur le sol, ou à proximité immédiate, mais désignent les urnes juvéniles, produites avant la transition ontogénique. Alors que les urnes du bas sont reliées au limbe foliaire par des vrilles peu souples et l'ouverture de ces urnes est orientée vers l'axe de la plante, chez les urnes du haut, les vrilles se « circonvolutionnent » au moins une fois et leur ouverture est généralement orientée vers l'extérieur de la plante, probablement du fait de l'installation de la vrille qui exerce une torsion sur l'urne (Figure 6).

Cette transition ontogénique s'accompagne également de modifications des caractéristiques du piège et une partie de ce travail de thèse a consisté à caractériser ces changements développementaux et fonctionnels.

Distribution et écologie

Le genre *Nepenthes* comprend environ 120 espèces (McPherson, 2009), principalement réparties en Asie du Sud-Est, avec Bornéo et Sumatra comme centres de diversité. Les limites de l'aire de distribution sont Madagascar à l'ouest, l'Inde au Nord, l'Australie et la Nouvelle-Calédonie pour le sud et l'est, les espèces décrites dans ces régions sont peu nombreuses (Figure 7).

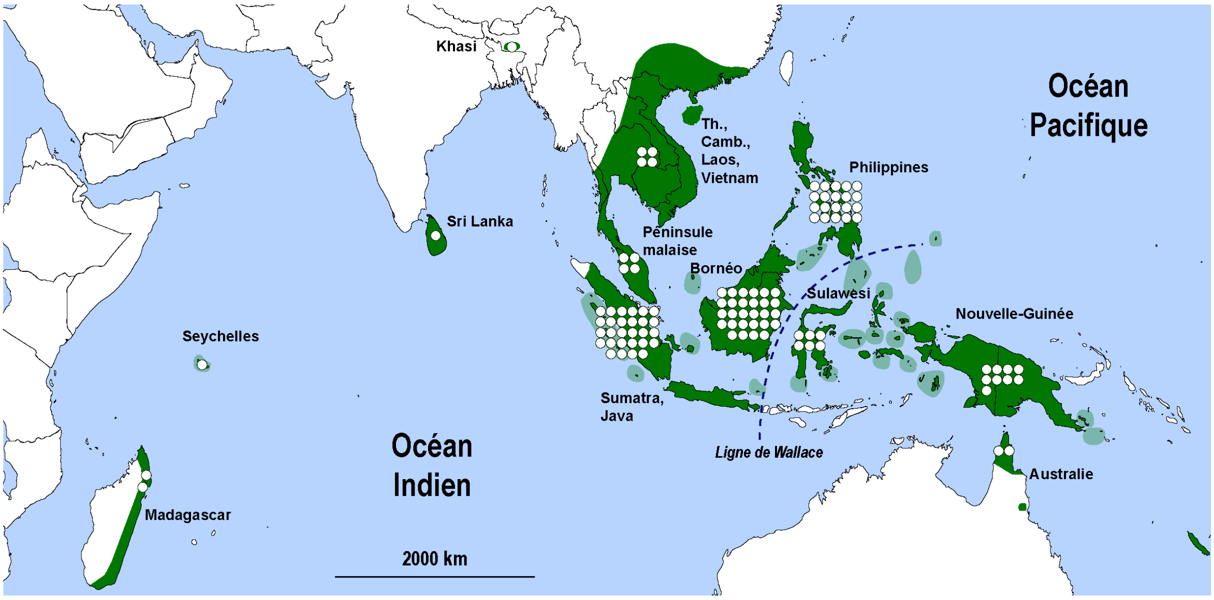

Figure 7 : Distribution et centres de diversité du genre *Nepenthes*. Les 120 espèces qui composent le genre *Nepenthes* sont principalement réparties en Asie du sud-est (en vert), avec les îles de Bornéo, de Sumatra et des Philippines comme centres de diversité et d'endémisme. Les points indiquent le nombre d'espèces strictement endémiques des foyers de colonisation inscrits sur la carte.

Il existe une grande diversité écologique au sein du genre, les *Nepenthes* croissent des plaines côtières ouvertes aux étages montagnards, des forêts de landes et/ou marécageuses, aux clairières de forêts plus denses. Leur distribution altitudinale, s'étale du niveau de la mer à 3400 m et les conditions climatiques auxquelles les *Nepenthes* sont soumises varient largement (Adam *et al.*, 1992, Cheek & Jebb, 2001). Les cultivateurs distinguent d'ailleurs dans leurs cultures les espèces de plaine des espèces de montagne, les dernières ayant besoin de fraîcheur hivernale à latitude d'origine comparable (J.-J. Labat, *comm. pers.*). Cette diversité écologique est couplée à une grande diversité morphologique des organes, et notamment des pièges (Figure 8).

Figure 8 : Un aperçu de la diversité morphologique du genre *Nepenthes*. De haut en bas et de gauche à droite : *Nepenthes mirabilis* var. *echinostoma*, une variété de *Nepenthes mirabilis* au péristome très élargi et uniquement présente au Brunei. *Nepenthes rafflesiana* var. *typica*, l'une des espèces modèles du genre. *Nepenthes albomarginata* dont la bande de trichomes clairs autour du péristome est vue comme une adaptation pour la capture spécifique de termites. *Nepenthes bicalcarata* et ses deux nectaires géants qui surplombent l'ouverture de l'urne. *Nepenthes ampullaria*, partiellement détritivore, produit des urnes du bas souvent tassées en « tapis », l'opercule est très réduit et forme un angle ouvert avec l'ouverture de l'urne. *Nepenthes rajah*, qui produit les urnes les plus volumineuses du genre. *Nepenthes aristolochioides* produit des urnes a la morphologie unique au sein du genre. Photographies Laurence Gaume, Wikimedia Commons.

Phylogénie du genre : état de l'art

Les phylogénies moléculaires actuellement disponibles (Meimberg *et al.*, 2000, Meimberg *et al.*, 2001, Meimberg & Heubl, 2006, Meimberg *et al.*, 2006) ne sont que peu résolutives et ne permettent pas de tester des hypothèses écologiques et évolutives concernant le genre. Plusieurs regroupements infra-génériques ont été proposés (Danser, 1928, Clarke, 1997) mais n'ont pas été retenus lors de la révision taxinomique complète du genre par Cheek et Jebb (Jebb & Cheek, 1997, Cheek & Jebb, 2001). Les travaux en taxinomie, bien que nombreux, ont été peu axés sur le regroupement en sous-genres qui refléterait les grandes lignes des relations évolutives au sein du genre. Ces travaux se sont surtout efforcés de décrire un nombre maximum d'espèces, et cette tendance reste très actuelle probablement du fait que les taxonomistes sont également des collectionneurs qui commercialisent leurs plantes (Figure 9). Si bien qu'il est difficile sur la base des connaissances actuelles en systématique, taxinomie et phylogénie du genre *Nepenthes* d'élaborer des scénarios évolutifs. Les seuls résultats qui semblent acquis sont que les espèces occidentales endémiques du Sri Lanka, des Seychelles et de Madagascar soient les plus basales, ces espèces sont soit apparues au moment de la formation de ces îles, soit elles se sont formées à l' issue d'une colonisation secondaire de ces îles (Meimberg & Heubl, 2006, Meimberg *et al.*, 2006).

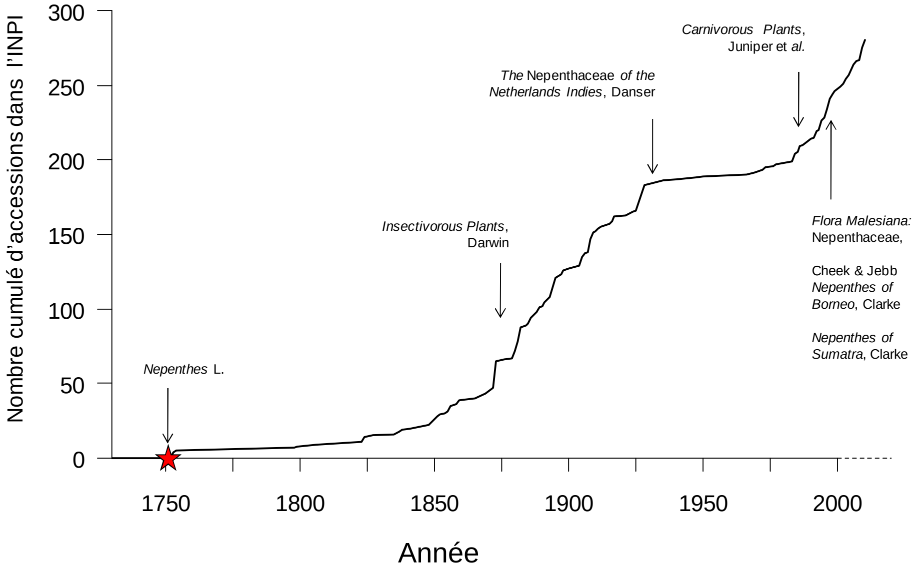

Figure 9 : Dynamique de descriptions des plantes du genre *Nepenthes* depuis la création du genre par Linné en 1753. Le graphique présente la somme cumulée du nombre de taxa (espèces, variétés, etc.) que recense l'*International Name Plant Index*. Depuis le début du siècle, le nombre de taxa décrits connait une deuxième phase d'expansion importante faisant suite aux nouveaux voyages d'exploration. Sont indiqués la date de creation du genre et les ouvrages majeurs. La dynamique du nombre d'espèces est comparable.

FONCTIONNEMENT DU PIÈGE DES *NEPENTHES*

Le piège des *Nepenthes* comprend de nombreuses adaptations physico-chimiques et morphologiques que nous allons détailler de façon exhaustive. Cette description rassemble tous les traits décrits liés à la carnivorie: nous verrons plus loin que l'assemblage de ces différentes composantes varie entre espèces et au cours de l'ontogénie des plantes. La description s'égrène de la partie apicale de l’urne jusqu’à la zone digestive, ce qui correspond peu ou prou à la séquence classique de capture.

Typologie du piégeage

Les termes reliés à la capture d’insectes dans ce manuscrit sont définis comme suit :

- *Attraction* : phase du piégeage qui amène les insectes à arriver à proximité immédiate de l’urne.
- *Capture* : phase du piégeage qui se déroule sur les parois externes du piège, sur le péristome qui borde l’ouverture de l’urne y compris, et qui fait pénétrer les insectes dans l’urne.
- *Rétention* : phase du piégeage qui se déroule à l’intérieur de l’urne et qui aboutit à sa mort dans l’urne. L’insecte est alors appelé *proie*.
- *Digestion* : ensemble des processus qui permettent à la plante d’intégrer dans son propre métabolisme les éléments dérivés des proies.
- *Type d’urne* : les différentes catégories d’urnes produites au cours de l’ontogénie.
- *Stratégie de piégeage* : combinaison pour une espèce et un type d’urne donné de mécanismes spécifiques impliqués dans le piégeage.

Opercule

La partie supérieure de l’urne, c'est-à-dire l’apex de la feuille modifiée, est surmontée d’un opercule. Cet opercule sécrète du nectar sur la face abaxiale, c’est-à-dire celle en regard de l’urne, plus ou moins abondamment selon les espèces (Figure 10-1).

L’angle que forme l’opercule avec le péristome est inférieur à 90° (et généralement à 60°) chez la plupart des espèces, et sa projection sur le péristome recouvre donc partiellement l’ouverture de l’urne. Deux hypothèses ont été avancées quant à une éventuelle fonction de l’opercule : (1) un rôle dans la protection de l’urne contre les précipitations, la dilution du liquide digestif et la perte éventuelle de proies capturées; (2) un rôle dans l’attraction des insectes par ses nectaires concentrés sur la face abaxiale qui positionne les des insectes dans une posture périlleuse au dessus de l’ouverture de l’urne.

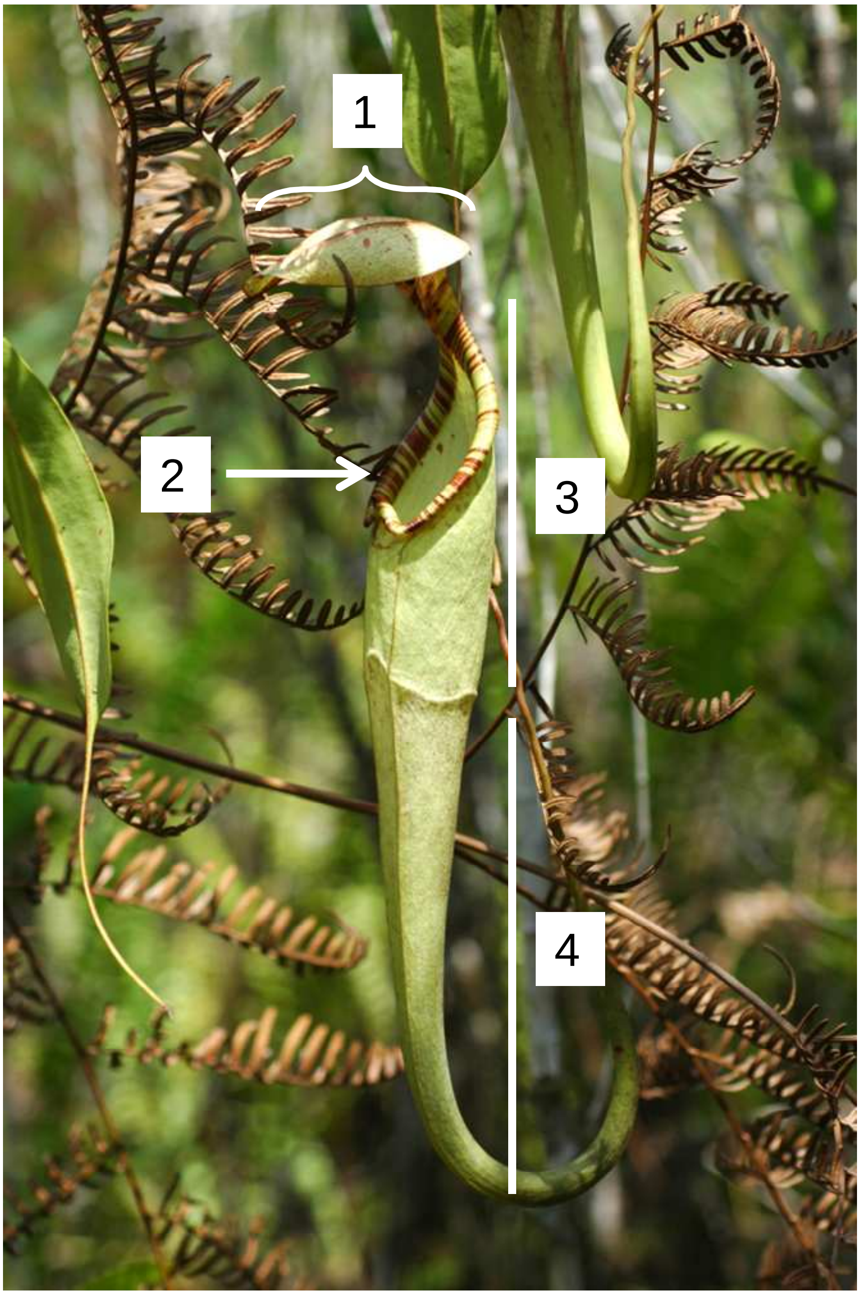

Figure 10 : Organisation générale d'une urne de *Nepenthes*. 1 = opercule nectarifère ; 2 = péristome nectarifère et odorifère chez certaines espèces ; 3 = zone conductive, généralement cireuse ; 4 = zone digestive. Un bourrelet, bien visible sur cette urne aérienne de *Nepenthes rafflesiana* var. *elongata*, sépare généralement les zones conductives et digestives.

Le péristome

Le péristome est la collerette dentée qui entoure la bouche de l'urne (Figure 10-2). Anatomiquement, il est constitué de cellules épidermiques modifiées (Owen & Lennon, 1999) qui se recouvrent partiellement et qui forment des stries disposées radialement, qui forment vers l'intérieur de l'urne des « dents ». Les stries et les dents du péristome présentent une certaine variabilité au sein du genre. Chez *Nepenthes villosa* les dents mesurent par exemple plusieurs centimètres et les stries sont marquées et creusent de profonds sillons. Chez *N. northiana* et *N. jacquelinae*, les dents sont en revanche bien moins marquées mais le péristome est très élargi. Chez *Nepenthes lowii* et *N. khasiana,* le péristome est quasi-absent, et réduit à une bande lisse de moins d'1 mm qui ceint l'urne.

Entre les dents ou à l'extrémité, du nectar est sécrété. Outre son attractivité intrinsèque, il est impliqué dans le piégeage des insectes. Une solution de sucre a des propriétés hygroscopiques : elle favorise la condensation de l'humidité atmosphérique et diminue le taux d'évaporation (Bauer *et al.*, 2008). De fait, ce péristome, apparaît souvent luisant, au moins chez certaines espèces (*Nepenthes rafflesiana* par exemple). Du fait du chevauchement partiel des cellules du péristome, ce dernier possède une structure anisotrope et glissante. La présence d'une pellicule d'eau favorise un effet d'*aquaplaning* sur les insectes (Bohn & Federle, 2004).

En plus de ces propriétés mécaniques, le péristome de certaines espèces produit des odeurs florales impliquées dans l'attraction d'insectes volants et qui contribuent notamment à la capture d'un large spectre d'insectes chez *Nepenthes rafflesiana* (Di Giusto *et al.*, 2008, Di Giusto *et al.*, 2010).

La zone conductive

Plus bas, se trouve une zone conductive qui constitue environ la moitié supérieure de l'urne chez la plupart des espèces (Figure 10-3). Cette zone est recouverte chez la plupart des espèces d'un manteau de cire épicuticulaire dont l'aspect est blanchâtre. Cette cire est depuis longtemps considérée comme un élément clé du piégeage (Juniper & Burras, 1962). La cire cuticulaire, vue comme une adaptation acquise lors de la terrestrialisation, est omniprésente chez les végétaux terrestres mais elle est rarement produite en quantité (Barthlott, 1990, Juniper, 1995, Barthlott *et al.*, 1998, Kunst & Samuels, 2003, Müller & Riederer, 2005). La cire des *Nepenthes* est composée d'une double couche dont la zone épicuticulaire est principalement composée de longue chaînes de carbone (triacontanal C_{30} et dotriacontanal C_{32}) Riedel, Eichner, et Jetter, 2003; Riedel *et al.*, 2007) lui conférant à l'échelle microscopique une conformation autoorganisée en paillettes, orientées principalement orthogonalement à la surface (Gorb et al. 2005) et faiblement accrochées à l'épiderme de l'urne (Riedel *et al.*, 2003, Riedel *et al.*, 2007).

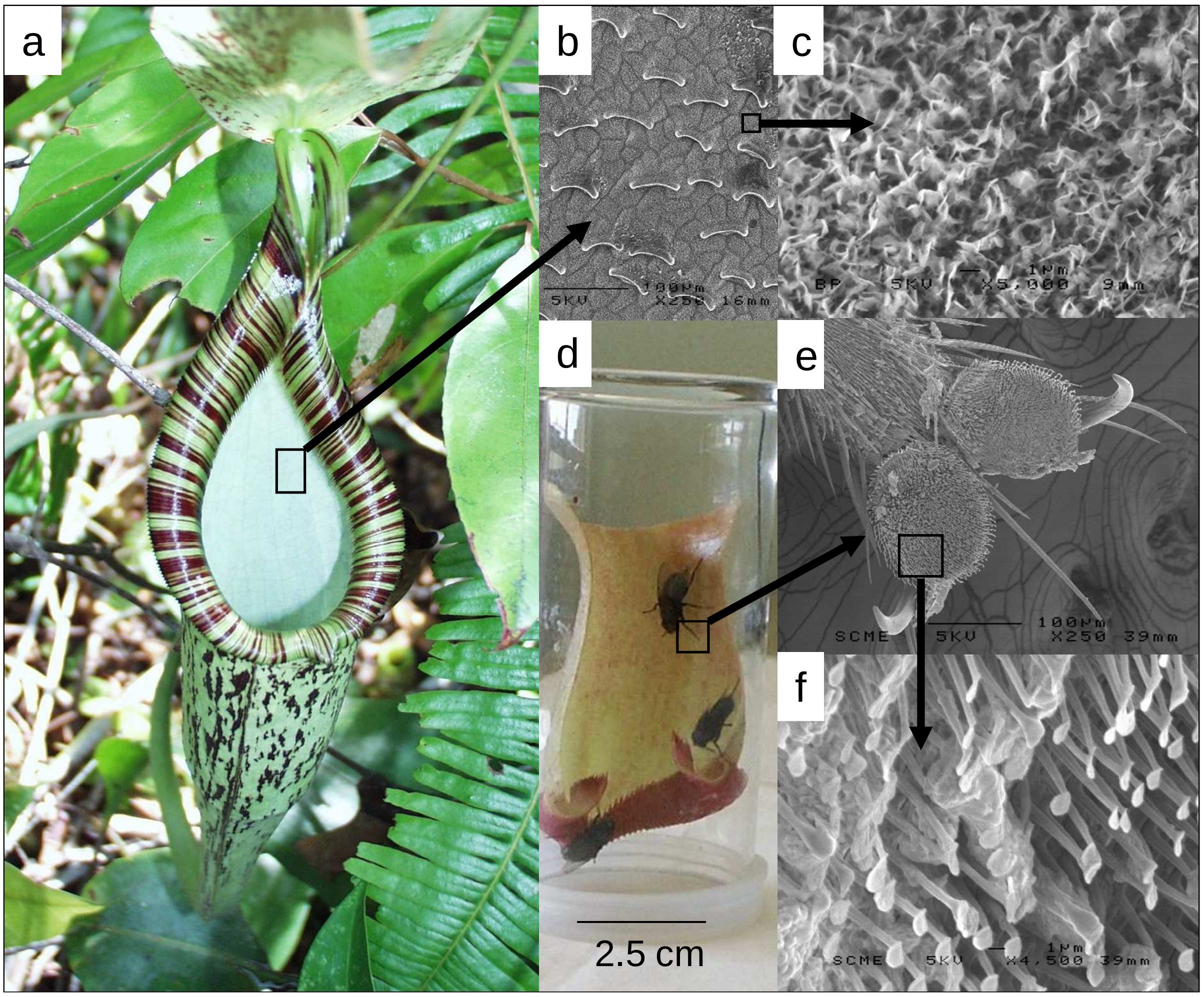

Figure 11 : Rôle de la cire dans la capture des insectes (Gaume *et al.*, 2002, 2004). La zone cireuse qui recouvre la partie supérieure de l'urne (a) est composée de stomates modifiés en « tuiles » dirigées vers le bas (b) et recouverte d'un manteau de cire. Ces cristaux de cire (c) sont aisément détachables et forment la partie superficielle de la zone cireuse. Le dispositif expérimental (d) montrant une mouche entrain de « déraper » sur la zone cireuse. Les systèmes d'attachement des mouches (e, f) montrent les deux pelotes adhésives soyeuses contaminées par la cire. Les cristaux de cire se détachent au contact des pelotes de l'insecte et semblent s'être dissous en une pâte amorphe qui piège les soies et les empêche de fonctionner comme spatules souples et adhésives.

Cette conformation des paillettes offre non seulement une structure rugueuse qui diminue les points d'attachement des insectes mais également une structure non-cohésive qui cède sous l'action des pattes d'un insecte, ce qui facilite la chute de celui-ci dans l'urne puis sa rétention.

Cette cire aisément détachable (Juniper & Burras, 1962) contamine les systèmes d'attachement des insectes (Gaume *et al.*, 2004) et les empêchent d'adhérer aux parois. Les systèmes d'attachement des mouches sont faits de très nombreuses soies (Federle, 2006) au bout desquelles est sécrétée un fluide adhésif (Gorb, 1998). Les cristaux de cire qui se détachent des *Nepenthes* sont en en fait dissouts au contact des pelotes des mouches et probablement du micro-fluide qu'elles sécrètent au niveau de leurs soies. La cire prend alors une conformation amorphe qui englobe les soies des *pulvilli* (Figure 11), les empêchant de fonctionner comme spatules souples et adhésives (Gaume et al. 2004). Après contamination par la cire, les mouches ne sont plus capables de monter sur la paroi de l'urne, ni sur une paroi en verre.

Les cristaux de cire ne constituent pas le seul obstacle à la remontée des insectes. Une proportion importante de fourmis parvient à remonter les parois d'une zone cireuse retournée à 180°. Des observations au microscope électronique à balayage ont montré que la zone sous-jacente à la cire forme une surface tuilée anisotrope (Figure 11-b) où chaque tuile de forme convexe, qui proviendrait de la modification d'une cellule de garde stomatique (Lloyd, 1942), n'offre aucune prise aux griffes de l'insecte lorsque l'urne est dans sa position normale, ce qui n'est pas le cas lorsqu'elle est en position inversée (Gaume *et al.*, 2002).

Le fonctionnement de la zone conductive des urnes des *Nepenthes*, qui repose sur des micro-rugosités, des surfaces anisotropes, des paillettes non-cohésives qui se transforment en pâte collante au contact des insectes est finalement plus complexe qu'imaginée initialement. Les proies sont digérées dans la zone digestive, immédiatement située en dessous la zone conductive et remplie du liquide digestif (Figure 11-e,f)

Liquide digestif

Ce liquide est sécrété lors de la formation de l'urne, avant son ouverture. La caractérisation des enzymes digestives des *Nepenthes* est un des axes historiques de recherche sur le genre (Jentsch, 1972, Tökés *et al.*, 1974). Ce liquide est acidifié par l'activité de pompes à protons (An *et al.*, 2001). Le pH des liquides digestifs décrits varie entre 1,5 et 5,5 (Clarke, 1997), vraisemblablement en lien avec les différentes stratégies d'obtention de nutriments au sein du genre (Moran *et al.*, 2010). Pour les liquides les plus acides, le pH correspond à l'optimum des enzymes de type protéases (An *et al.*, 2002a) et chitinases (An *et al.*, 2002a, Eilenberg *et al.*, 2006, Hatano & Hamada, 2008), qui décomposent les protéines et la chitine des insectes (An *et al.*, 2002b).

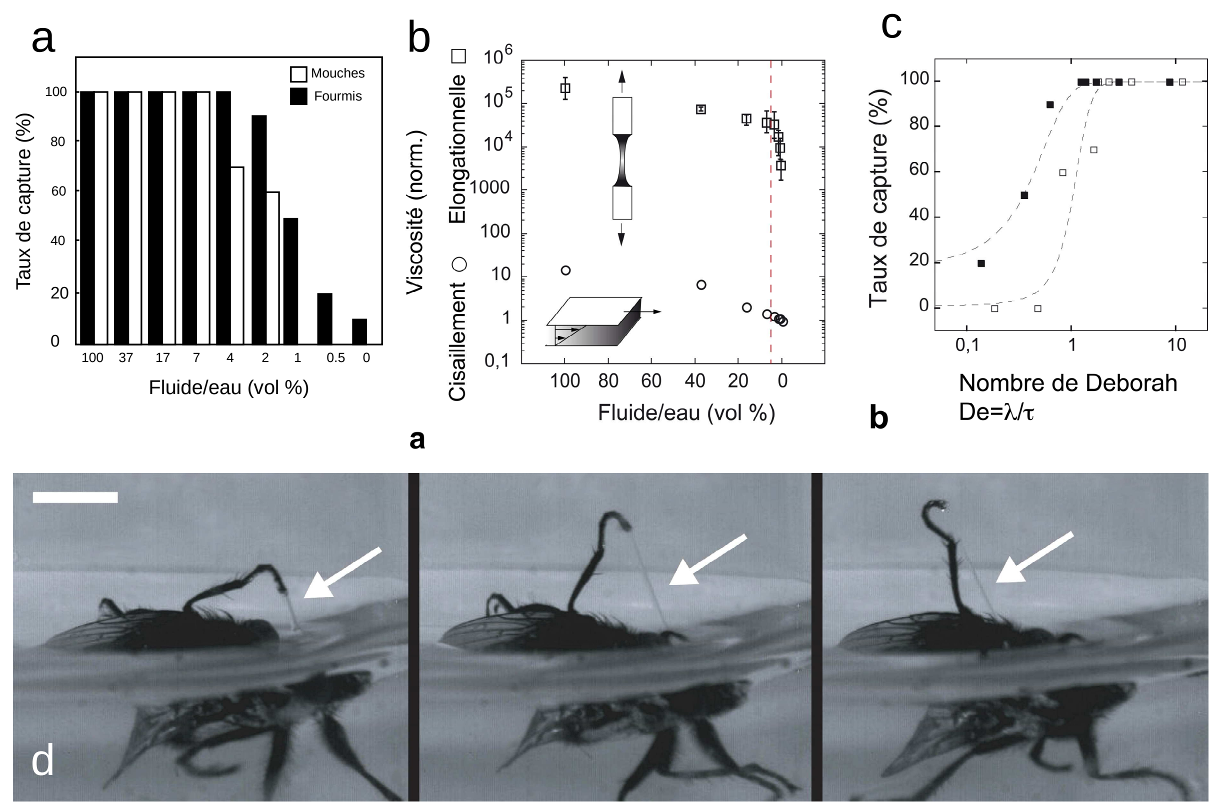

Figure 12: Rôle des propriétés viscoélastiques du fluide digestif dans la capture des insectes d'après Gaume & Forterre (2007). (a) Taux de capture des mouches (en blanc) et des fourmis (en noir) pour des fluides de *Nepenthes rafflesiana* de plus en plus dilués par l'eau. (b) Viscosités élongationnelle (carrés) et de cisaillement (cercles) du fluide. La viscosité élongationnelle ou élastique est obtenue en mesurant le temps de rupture d'un filament liquide. Le trait rouge indique la concentration au-dessus de laquelle le taux de capture est de 100 %. Seule la viscosité élastique du fluide reste élevée lorsque le fluide est dilué. Tout comme ses propriétés rétentives, elle ne diminue sensiblement qu'à de fortes dilutions. (c) Taux de capture en fonction du rapport entre le temps de relaxation élastique λ du fluide et le temps typique de mouvement des insectes T dans le fluide (appelé nombre de Deborah, $De = \lambda / T$). Le fluide digestif piège les insectes tant que leurs mouvements sont trop rapides pour laisser les forces élastiques du fluide se relaxer ($\lambda > T$). Ainsi plus l'insecte se débat, plus le milieu lui apparait « solide ». (d) Séquences d'un film pris à la caméra rapide montrant comment une mouche tombée dans le fluide viscoélastique est retenue par des filaments de longue durée de vie.

Les produits de digestions : peptides, ammoniac, et acides aminés sont absorbés par les glandes digestives (Schultze *et al.*, 1999) qui se différencient à partir de cellules épidermiques lors du développement de l'urne (Owen & Lennon, 1999). De courtes chaînes carbonées (C_2) dérivées d'acides aminés sont également directement intégrées au métabolisme de *Nepenthes insignis* (Rischer *et al.*, 2002).

Le liquide digestif est également composé de polymères géants (Gaume et Forterre 2007) de nature polysaccharidique (Alain Heyraud, *com. pers.*) aux propriétés viscoélastiques qui jouent un rôle physique dans la rétention des proies.

L'urne comme habitat : rôle dans la digestion

Les plantes à urnes forment également des phytothelmes (Frank, 1983), des structures végétales creuses qui retiennent l'eau. Ces microcosmes abritent une communauté bactérienne et une microfaune ou *infauna* impliquée dans la dégradation et la digestion des proies des plantes à urnes. Chez *Darlingtonia californica*, il ne semble pas y avoir d'enzymes digestives et c'est cette *infauna* qui assure seule la dégradation des proies. L'infauna des plantes carnivores à urnes forme un microécosystème dont on peut contrôler les paramètres et est devenue un sujet en soit d'écologie des communautés et d'écologie fonctionnelle (Clarke & Kitching, 1993, Cresswell, 2000, Kneitel & Miller, 2002, Kneitel & Miller, 2003).

Les urnes des *Nepenthes* sont par ailleurs habitées par une faune qui leur est inféodée : des acariens (Fashing & Chua, 2002), diverses larves de diptères, incluant des moustiques (Clarke, 1997), des araignées inféodées à l'urne (Clarke, 1997, Pelloux-Prayer, 2007), voire des fourmis (Beccari, 1904, Clarke & Kitching, 1995) ou même des grenouilles (Das & Haas, 2010) et des crustacés (Clarke, 1997 ; *pers. obs.*) qui elle aussi peut être partiellement impliquée dans la digestion des proies.

Implication du liquide digestif dans la rétention

Bruce Salmon propose pour la première fois en 1993 dans la *Carnivorous Plant Newsletter,* que le fluide digestif de *Nepenthes inermis,* décrit comme très visqueux, pourrait être impliqué dans la rétention du fluide lui-même et de ses victimes au sein du piège en cas de fortes pluies[3] (Salmon, 1993).

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[3] "[...] one wonders how the pitcher manages to keep its prey from being washed out when it rains. The answer lies in the pitcher fluid itself. The fluid is thick and exceptionally viscous compared to most *Nepenthes*. You can pour some from a pitcher held several feet high and it will keep in an unbroken stream to the ground. Thus when it rains the pitcher sheds the excess water over its rim but its content are secure."

Chez certaines espèces, dont notre modèle *Nepenthes rafflesiana*, le liquide digestif crée des filaments lorsque l'urne est renversée ou lorsque l'on y plonge le doigt et qu'on l'en ressort (Figure 12). Ce phénomène ne peut s'expliquer seulement par la viscosité mais par une composante élastique du liquide qui joue un rôle central dans le piégeage. Gaume et Forterre ont montré, dans une étude à l'interface entre la biologie et la mécanique des fluides que le comportement physique de ce liquide jouait un rôle crucial dans la rétention des insectes vivants tombés dans l'urne. Cette étude a ouvert une bonne partie des travaux de recherche de ce doctorat (Gaume & Forterre, 2007).

Les propriétés physiques des fluides sont essentielles pour comprendre les mécanismes des grandes fonctions biologiques et physiologiques des organismes : le vol des insectes (Dudley, 2002, Sane, 2003), la motilité bactérienne (Schneider & Doetsch, 1974), la circulation sanguine (Ku, 1997). À l'échelle centimètrique et en deçà, la gravité, contrainte principale des organismes grands et lourds, s'estompe devant l'effet de la tension de surface et de la viscosité.

La viscosité est la résistance d'un fluide à la déformation. On distingue plusieurs types de viscosité, dont la viscosité linéaire et la viscosité élastique (ou extensionnelle). La viscosité linéaire est la résistance qu'oppose un fluide à son cisaillement, c'est-à-dire au passage d'un corps et qui augmente proportionnellement avec la vitesse de cisaillement. Le terme de viscosité est l'acceptation dans le langage courant de la viscosité linéaire: l'air est moins visqueux que l'eau, elle-même moins visqueuse que le miel et le bitume ; et un solide a une viscosité infinie.

Les fluides viscoélastiques combinent, quant à eux, une composante linéaire et une composante élastique. L'élasticité est la propriété qu'a un corps de revenir à l'état initial après une déformation. Un fluide viscoélastique a des propriétés visqueuses (il s'oppose au passage d'un corps) et élastique (il tend à revenir à l'état initial après déformation).

La viscosité de l'eau ne dépend pas de la vitesse du cisaillement, les seules contraintes de son écoulement sont des contraintes de cisaillement, et ces contraintes cessent immédiatement dès que cet écoulement est interrompu, ce fluide est dit newtonien. La plupart des fluides organiques ne sont pas newtoniens et dérogent aux trois propriétés précédentes. La viscosité peut ainsi diminuer avec la vitesse de cisaillement (par ex. le sang) ou augmenter (par ex. une solution de fécule de maïs dans de l'eau) et les fluides seront respectivement qualifiés de rhéofluidifiants et de rhéoépaissisants.

Les travaux de Gaume et Forterre ont mis à jour plusieurs éléments majeurs du piégeage chez l'espèce *Nepenthes rafflesiana* var. *typica* :

- la tension de surface du liquide est similaire à celle de l'eau et n'est donc pas impliquée dans la rétention des insectes ;

- le fluide digestif est environ 15 fois plus visqueux que l'eau pure au taux de cisaillement qui correspond à celui d'un insecte ;
- ce liquide est rhéofluidifiant : la viscosité diminue avec le taux de cisaillement.
- ce liquide est également très viscoélastique et c'est cette viscoélasticité qui est responsable de son important pouvoir rétentif ;
- l'efficacité de capture des insectes par le liquide reste forte tant que le temps de relaxation élastique du liquide est plus important que la période de mouvement de l'insecte. Ainsi à l'image des sables mouvants, plus un insecte se débat dans le liquide, plus la force de rétention élastique exercée est grande et donc plus il est piégé. Le piège viscoélastique est donc activé par le propre mouvement de la proie (Figure 12).
- ce liquide retient encore 100% des insectes lorsqu'il est dilué dans 95% d'eau (Figure 12).

Autres composants de l'attraction

Des motifs sur les parois externes de l'urne, notamment ceux visibles dans l'ultra-violet, domaine du spectre lumineux que les hyménoptères en particuliers perçoivent, sont impliqués dans la rétention (Moran, 1996, Moran *et al.*, 1999). Chez toutes les espèces de *Nepenthes* (Juniper et al. 1989), la surface externe de l'urne et dans une moindre mesure le reste de la plante est parsemé de nectaires extrafloraux impliqués dans l'attraction des insectes (Merbach *et al.*, 2001).

Stratégies alternatives d'absorption de nutriments

Plusieurs espèces de *Nepenthes* présentent des stratégies de piégeage dont les composantes n'entrent pas dans les schémas décrits ci-dessus. *Nepenthes ampullaria* se développe dans des forêts assez denses et présente un péristome étroit et plongeant abruptement vers l'intérieur de l'urne (Figure 13). Les débris végétaux retrouvés dans l'urne contribuent à la à hauteur de 35 % de l'azote foliaire de cette espèce, qui peut donc être considérée comme partiellement détritivore (Moran *et al.*, 2003).

Chez au moins trois espèces de montagne de Bornéo, l'angle entre le plan d'ouverture de l'urne et l'opercule est plus ouvert que chez la plupart des autres espèces, compris entre 80° et plus de 100°. Clarke *et al.* (Clarke *et al.*, 2009), puis Chin *et al.* (Chin *et al.*, 2010) ont proposé que cette caractéristique géométrique est adaptée à la morphologie de petites musaraignes arboricoles de la famille des Tupaiidae (*tree shrew*) qui, en se positionnant à califourchon sur l'urne pour consommer le nectar sécrété sur la face abaxiale de l'opercule sont dans une position idéale pour déféquer dans l'urne. Les fèces sont effectivement assimilés par l'urne (Chin *et al.*, 2010), ce qui, dans un piège carnivore, n'a rien d'inattendu.

PROBLÉMATIQUE

Historiquement les recherches sur le genre *Nepenthes* se dissocient en deux axes, l'un s'attache à obtenir une description morpho-taxinomique exhaustive du genre et l'autre se focalise sur l'écologie, le fonctionnement du piégeage, de la physiologie de la digestion, etc. d'une poignée d'espèces modèles.

Aucune étude à ce jour n'a essayé de comprendre si la diversification morphologique du genre *Nepenthes* avait un lien avec une diversité fonctionnelle et pouvait être expliquée par des pressions de sélection d'ordre écologique. On peut raisonnablement supposer que les milieux très variés que ces plantes carnivores colonisent, ainsi que leurs différents modes de vie (rampants, grimpants, épiphytes) les exposent à des environnements très contrastés en termes de proies disponibles. Cette diversité de l'entomofaune joue très probablement un rôle moteur dans la diversification morphologique et fonctionnelle des pièges des *Nepenthes*. Cette thèse s'emploie à tester cette hypothèse. La logique d'ensemble de ce travail peut être résumée en deux grands axes.

Quelle est la diversité du fonctionnement et de la production des pièges au sein du genre ?

Dans un premier temps nous avons testé si les systèmes de piégeages différaient au sein du genre et si leurs efficacités variaient selon le type d'insectes rencontrés. Pour cela, nous avons mis en évidence chez un échantillon d'espèces les paramètres physico-chimiques clés telles que la couche cireuse glissante et le liquide digestif viscoélastique du système de piégeage. Nous avons quantifié ces composantes du piégeage chez de nombreuses espèces. Nous avons ensuite comparé entre espèces de *Nepenthes* l'efficacité relative de ces caractères sur différents types de proies (insectes volants et rampants) en serre, et sur le terrain afin de tester l'hypothèse selon laquelle les spectres de proies rencontrées par les *Nepenthes* pouvaient exercer une pression de sélection sur l'évolution des stratégies de piégeage au sein du genre.

Nos observations de terrain nous ont également amené à mettre en évidence une stratégie de piégeage alternative qui implique un mutualisme plante-fourmi. Cette étude reflète également la diversité des stratégies de piégeage des *Nepenthes*.

Par ailleurs, la production d'urnes implique nécessairement un coût pour les plantes. On s'attend donc à ce qu'il existe une plasticité dans la production d'organes carnivores qui permette de moduler l'investissement dans le compartiment carnivore selon les besoins saisonniers, ontogéniques, et la disponibilité variable de ces nutriments-clés, soit sélectionnée et/ou maintenue (Ellison *et al.*, 2003). Nous avons donc également suivi le développement des espèces du Brunei et les contraintes qui s’exercent sur la production des urnes extrinsèques à la relation « prédateurs »/ proies. Cette analyse nous a amené à étudier comment la production d’urnes pouvaient varier pendant la phase de reproduction de la plante.

Quelle est l’histoire évolutive du genre et des caractères clés du piégeage et quels rôles y joue l’écologie des espèces et l’ontogénie ?

Dans un deuxième temps nous avons essayé de comprendre l’évolution des caractères clés du piégeage, précédemment identifiés comme tels, à l’échelle du genre. Nous avons tenté d’améliorer les phylogénies moléculaires existantes. Nous avons retracé l’évolution de ces caractères sur ces phylogénies. Le but était de voir s’il existait des *trades-offs* entre caractères. Nous avons également étudié les associations entre l’écologie des espèces et les différentes stratégies de piégeage afin de tester si ces différents caractères ont évolué en réponse à des pressions de sélections différentes. Cette phylogénie nous a également permis d’étudier l’origine évolutive des caractères étudiés et le rôle de l’ontogénie dans l’évolution des caractéristiques des urnes. Comme nous l’avons évoqué dans la description du genre, la transition ontogénétique observée lors de la production d’urnes du haut s’accompagne souvent de modifications de forme et de structure des urnes. Nous avons donc recensé ces changements au sein du genre et étudié l’évolution de ces transitions dans le cadre de nos phylogénies. Cela nous a permis de tester si les changements de formes des urnes s’accompagnaient toujours des mêmes modifications au niveau de la zone cireuse. Nous avons également testé si les transitions ontogénétiques reflétaient certaines transitions évolutives.

TERRAINS D'ÉTUDE ET ESPÈCES ÉTUDIÉES

Le travail expérimental de cette thèse porte à la fois sur quelques espèces modèles étudiées sur le terrain et sur une collection d'espèces cultivées en serre.

Le Conservatoire National des Plantes Carnivores

À Peyrusse-Massas, au nord d'Auch dans le Gers, Jean-Jacques Labat possède la plus grande collection mondiale de plantes carnivores qui regroupe 450 espèces. Le genre *Nepenthes* y figure en bonne place avec plus de 110 espèces, variétés et hybrides horticoles. Nous avons eu la chance de collaborer avec J.-J. Labat et d'accéder à cette collection. Au cours de six missions de 2007 à 2010, nous avons pu accéder à l'essentiel des espèces du genre, mises en croissance dans différentes conditions de culture, chauffées en hiver et présentant une hygrométrie supérieure à 80%.

Terrains d'étude au Brunei Darussalam

Les études de terrain ont toute été réalisées dans le sultanat de Brunei Darussalam, au nord-est de l'île de Bornéo. Nos missions sur le terrain ont été effectuées sur deux sites principaux.

Le site de Labi Road (4°29.823' N, 114°27.576' E), est situé en bordure d'une forêt marécageuse à Dipterocarpaceae dominée par *Shorea albida* et d'une forêt de lande dominée par les fougères du genre *Gleichenia* (*« mixed peat swamp heath forest »*, Figure 13). Ce terrain est fréquemment inondé, avec des mares temporaires. Il abrite une importante densité de *Nepenthes ampullaria*, *N. gracilis, N. bicalcarata*, *N. rafflesiana* var. *elongata* et var. *gigantea*. Toutes les expérimentations ont été effectuées dans une zone à peu près circulaire d'environ 3 hectares.

Le site dit de *Tutong,* (04°44.520' N, 114°35.668' E) est une bande côtière de sables blancs (les *pasirs puteh* pour les malais), en bordure de *keranga,* forêt dégradée de Bornéo. Ce site, constitué d'une épaisse couche de sable drainant mais qui repose sur un fond d'argile imperméable, est donc fréquemment inondé (Figure 13). Le site est ponctué de quelques bosquets mais est globalement très ouvert. La végétation est dominée par des arbustes des genres *Melastomata* et *Syzygium,* et des fougères du genre *Gleichenia* (Di Giusto *et al.*, 2008). On y trouve également de nombreuse plantes à fourmis épiphytes du genre *Dischidia*.. Ce site présente une grande abondance de *Nepenthes rafflesiana* var. *typica* et de *Nepenthes gracilis*, dont nous détaillerons plus loin la biologie, ainsi qu'une station où *Nepenthes rafflesiana* var. *typica* et var. *elongata* poussent en sympatrie. Toutes les expérimentations ont été effectuées dans une zone d'environ 10 hectares (500 x 200 m).

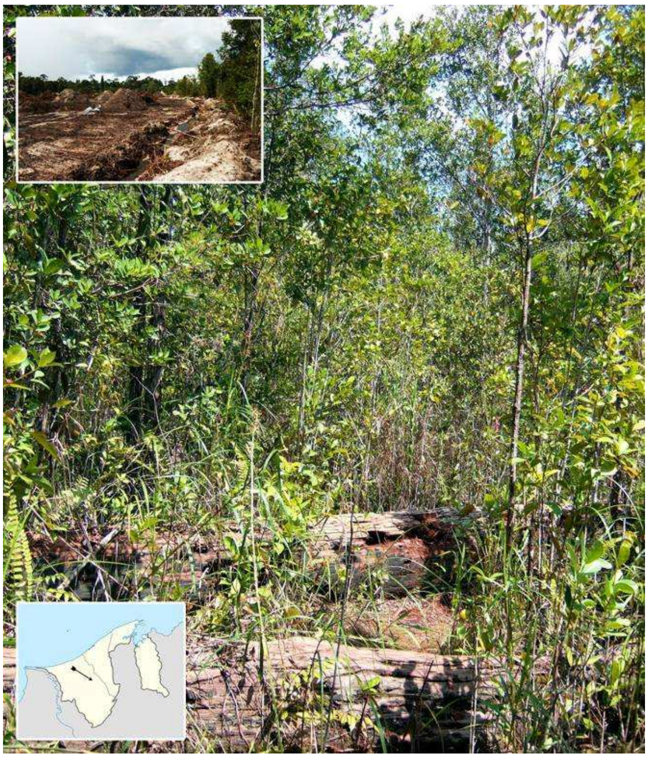

Figure 13: Le terrain de *Labi Road.*

Le terrain d'étude de *Labi road* 4° 29' N, 114° 27' E (médaillon bas). Ce terrain est constitué d'une forêt mixte marécageuse, souvent inondée et en bordure d'une forêt plus haute. Dans cette zone, plutôt unique, cohabitaient *Nepenthes ampullaria*, *bicalcarata*, *gracilis*, *rafflesiana* var. *gigantea*. En médaillon, la meme zone aujourd'hui, après prospection pétrolière (Photographie Ulmar Grafe).

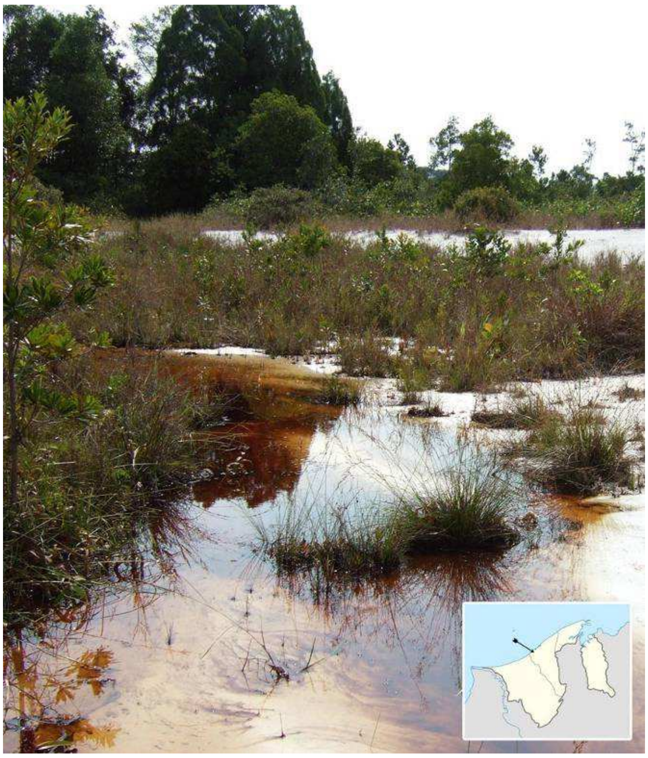

Figure 14 : Le terrain de Tutong

Le terrain d'étude de *Tutong* 4°44'N, 114°35'E (médaillon en bas à droite). Ce terrain est une bande côtière de sable blanc (les *pasir puteh* pour les malais), souvent inondée et en bordure d'une *heath forest*. A vol d'oiseau la mer de Chine est à un kilomètre. Les températures et l'insolation sont très fortes. La végétation est arbustive et abrite en nombre, *Nepenthes rafflesiana* et *gracilis* et des myrmécophytes du genre *Dischidia*.

Description des espèces étudiées sur le terrain

Nepenthes gracilis Korth. est sans doute l’espèce de *Nepenthes* la plus fréquemment rencontrée dans les milieux ouverts au Brunei et plus largement à Bornéo, Sumatra, en Malaisie péninsulaire et au Sulawesi principalement en plaine mais s’étend jusqu’à environ 1000 m (Clarke, 1997). Cette espèce produit de nombreux axes rampants qui peuvent s’élever sur un support en forêt, mais qui donne une forme rampante voire buissonnante en milieu ouvert. Les urnes terrestres et aériennes ont une forme similaire, leur production est prolifique, et le péristome est très réduit (Figure 15).

Nepenthes bicalcarata Hook f. est considérée comme la liane qui atteint les hauteurs les plus hautes au sein du genre *Nepenthes*, elle se développe jusqu’à une vingtaine de mètres de hauteur. Elle est endémique du nord-ouest de Bornéo, habitant en particulier les forêts denses et marécageuses ainsi que les bordures de celle-ci et de la forêt de landes (*heath forests*) en dessous de 1000 m d’altitude. Elle produit de grandes feuilles, probablement les plus grandes du genre ainsi que de nombreuses urnes longévives (Clarke, 1997). Ses urnes possèdent plusieurs nectaires dont deux géants en forme de dents et qui surplombent l’urne (Figure 15). Ses vrilles creuses sont habitées par la fourmi *Camponotus schmitzi* dont l’interaction mutualiste avec la plante-hôte sera discutée plus loin.

Nepenthes ampullaria Jack est une espèce de plaine qui affectionne les forêts denses de Thaïlande, Sumatra de Malaisie Péninsulaire, de Bornéo et de Nouvelle-Guinée (Cheek & Jebb, 2001). À Bornéo, on la trouve souvent en sympatrie avec *Nepenthes bicalcarata*. Elle produit des axes lianescents d’une quinzaine de mètres. À sa base, elle produit des tapis d’urnes, de dimensions modestes relativement aux autres espèces (Figure 15). Ces urnes ne sont ni cireuses, ni viscoélastiques, ce qui peut-être vu comme une adaptation à son régime partiellement détritivore. Les axes lianescents ne produisent que très rarement des urnes aériennes, mais plutôt des groupes d’urnes « terrestres » espacées de quelques mètres de part et d’autre de cet axe.

Dix variétés de *Nepenthes rafflesiana* Jack sont distribuées à Bornéo, Sumatra et sur la péninsule malaise (Clarke, 1997, Cheek & Jebb, 2001). Quatre d’entre elles, qui diffèrent par leurs stratégies de piégeage et la morphologie de leurs urnes (Di Giusto *et al.*, 2009, Gaume & Di Giusto, 2009), sont présentes au Brunei : *Nepenthes rafflesiana* var. *typica*, var. *nivea*, var. *elongata* et var. *gigantea* (Figure 16). *Nepenthes rafflesiana* est l’espèce modèle de l’équipe travaillant sur les plantes carnivores au laboratoire AMAP chez laquelle a été décrite les changements ontogéniques et le rôle de la surface cireuse (Di Giusto *et al.*, 2009, Gaume & Di Giusto, 2009), la viscoélasticité du liquide digestif (Gaume & Forterre, 2007) et les composants volatils formant le piège olfactif de la plante (Di Giusto *et al.*, 2010). *Nepenthes rafflesiana* présente des variétés différant par leurs patrons fonctionnels. Alors que *Nepenthes rafflesiana* var. *elongata* présente toujours une zone cireuse, *Nepenthes rafflesiana* var. *typica* la perd lors de la production des urnes du haut (Gaume & Di Giusto, 2009).

Figure 15 : Les *Nepenthes* du Brunei Darussalam (1/2)

Figure 16 : Les *Nepenthes* du Brunei Darussalam (2/2)

Chapitre I

DIVERSITÉ DES STRATÉGIES DE PIÉGEAGE ET DU DÉVELOPPEMENT DE QUELQUES ESPÈCES DE *NEPENTHES*

Chapitre I.1

DIVERSITÉ FONCTIONNELLE DES STRATÉGIES DE PIÉGEAGE DES PLANTES CARNIVORES DU GENRE *NEPENTHES*

Les découvertes scientifiques récentes de l'équipe ont changé notre vision du piège des *Nepenthes* : le mécanisme de la zone cireuse de l'urne dans le piégeage a été éclairci et les propriétés viscoélastiques du liquide digestif et son rôle dans la rétention des insectes ont été mis en évidence (Gaume *et al.*, 2004, Gaume & Forterre, 2007, Di Giusto *et al.*, 2008, Di Giusto *et al.*, 2009, Gaume & Di Giusto, 2009b). Devant une collection d'espèces du genre, la diversité morphologique est évidente et laisse supposer qu'elle est également associée à une diversité fonctionnelle. Par exemple, le caractère viscoélastique, décrit chez *Nepenthes rafflesiana* var. *typica* est-il un cas isolé ou est-il largement présent au sein du genre ? Quels sont les rôles respectifs de la zone cireuse et du liquide viscoélastique dans le piégeage des insectes ? Peut-on identifier différentes stratégies de piégeage à l'échelle du genre ? La zone cireuse et le liquide viscoélastique sont-ils des mécanismes efficaces pour tous les types d'insectes ? Si non, l'entomofaune du milieu peut-elle exercer une pression de sélection sur les stratégies de piégeage ? Toutes les stratégies sont-elles possibles ? Si non, quelles sont les contraintes qui s'exercent sur la production de ces mécanismes de piégeage ? Le **Manuscrit I.1** teste et amène des éléments de réponse à ces questions sur un sous-jeu d'espèces représentatif du genre.

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SLIPPERY OR STICKY? FUNCTIONAL DIVERSITY IN THE TRAPPING STRATEGY OF *NEPENTHES* CARNIVOROUS PLANTS

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Abstract

1. The pitcher-shaped leaves of *Nepenthes* carnivorous plants have been considered as pitfall traps that rely strictly on slippery surfaces and more specifically on a waxy coating to capture insects. But a recent study has shown in *N. rafflesiana* that the viscoelasticity of the digestive fluid found inside the pitchers also plays a key but cryptic role. We used insect bioassays and physical measures to determine whether *Nepenthes* species exhibit diverse trapping strategies. **2.** We tested 23 taxa, including 21 species (8 lowland and 13 mountain species), for the amount of wax on their pitcher walls and the viscoelasticity of their digestive liquid, i.e. two insect-trapping devices with unknown occurrence in the genus. In 12 of these species we compared the retention efficiency of wax and viscoelastic fluid on ants and flies. **3.** Our results show that species vary greatly with regard to the amount of wax produced and that at least two-thirds use viscoelastic fluids. We also show that both characters contribute significantly to insect trapping but that wax seems to be more efficient at trapping ants and that viscoelasticity seems to be key in trapping both types of insect, but is more efficient than wax on flies. Trap waxiness and fluid-viscoelasticity are inversely related, suggesting that the plants are faced with an investment trade-off. In our sample, only 25% of the lowland species, which typically have to cope with an ant-rich fauna, had a “viscoelastic” fluid whereas 92% of the mountain species facing prey fauna impoverished in ants but not in flying insects had a “viscoelastic” fluid. **4.** We conclude that *Nepenthes* insectivorous plants often employ a viscoelastic trapping strategy and should no longer be considered as simple pitfall plants with slippery traps. We suggest that the entomofauna specific to the plant’s habitat exerts selective pressure, encouraging one trapping strategy at the expense of the other. Further comparative studies on the ecology and prey fauna of “viscoelastic” *Nepenthes* versus “waxy” *Nepenthes* will be needed to test this hypothesis.

Key-words: Digestive fluid, insect capture, investment trade-off, leaf wax, pitcher plants, viscoelasticity.

Introduction

Carnivorous plants circumvent the nutrient shortage characterizing the habitats they colonize by deriving key nutrients from arthropods which they attract, trap and digest in specialized leaves (Juniper *et al.*, 1989, Ellison & Gotelli, 2001). Nepenthes (Caryophyllales: Nepenthaceae) is a climbing and carnivorous plant genus characterized by leaves modified as pitcher traps (Fig. 1). It encompasses more than 100 species, mainly distributed in southeastern Asia, with Borneo and Sumatra islands as hotspots of diversity (Clarke, 1997, Cheek & Jebb, 2001, Clarke, 2001, McPherson, 2009). Nepenthes species show a great diversity of pitcher morphologies and colonize various habitats including coastal lowlands, cliff habitats and high-altitude forests with a high rate of endemism (Clarke, 1997, McPherson, 2009). Such morphological diversity could stem from their adaptation to different arthropod fauna, and reflect differences in trapping strategies. But, it is not known whether these pitcher plants are also functionally diverse and whether such functional diversity can be linked to ecological characteristics of their environment. *Nepenthes* species are known to vary in their arthropod prey assemblage (Kato *et al.*, 1993, Adam, 1997, Merbach *et al.*, 2002) and even in their N-sequestration strategies (Moran *et al.* 2010) with some outlying species moving away from a purely carnivorous habit by deriving part of their nitrogen from leaf detritus (Moran *et al.* 2003), vertebrate faeces (Clarke *et al.*, 2009, Chin *et al.*, 2010), or from the nutritional service of a symbiotic hunter ant (Bonhomme *et al.* in press). The trapping strategy of strictly insectivorous species (the vast majority of these pitcher plants) has never been investigated in a comparative study within the genus.

Plants in the *Nepenthes* genus have long been thought to function as simple pitfall traps relying on slippery surfaces that decrease insect adhesion (Juniper & Burras, 1962, Juniper *et al.*, 1989, Gaume *et al.*, 2002, Gaume *et al.*, 2004, Gorb *et al.*, 2005) and wettable surfaces that cause insect aquaplaning (Bohn & Federle, 2004, Bauer *et al.*, 2009). But recently, in 2007, *Nepenthes rafflesiana* was shown experimentally to use another mechanism. It produces a digestive liquid partly made up of long-chain polymers, the viscoelastic properties of which have a marked effect on insect retention (Gaume & Forterre, 2007, Di Giusto *et al.*, 2008). Even when greatly diluted by water, the digestive liquid in *N. rafflesiana* has sufficient elastic properties to trap insects (Gaume & Forterre, 2007). This not only means that the digestive liquid might be crucial for the capture success of this tropical pitcher plant that is often subjected to heavy rains, but also that even species with a liquid viscosity similar to that of water may have unsuspected elastic properties that result in high trapping capacities. Therefore, the viscoelastic character of the digestive fluid might have remained cryptic in a number of species and could be far more widespread than expected in the *Nepenthes* genus. Interestingly, *N. rafflesiana* var. *typica* bears pitchers with a waxy zone and mainly traps ants during its juvenile phase; but as the plant ages the waxy zone is lost (Gaume & Di Giusto, 2009a) and the upper pitchers which are only produced in the adult phase contain a highly viscoelastic fluid that proves to be very efficient against flying insects (Di Giusto *et al.*, 2008). By contrast, the elongated traps of *N. rafflesiana* var. *elongata* keep their waxy zone throughout plant ontogeny and the plant mainly captures ants (Gaume & Di Giusto, 2009).

This casts doubts on the common belief that all *Nepenthes* species exhibit the same trapping strategy based on the slipperiness of their pitchers, and this study explores whether viscoelastic fluids are common among *Nepenthes* species. It also addresses the question of the cost of producing both a slippery waxy zone and a viscoelastic fluid and whether these two retentive devices have similar effects on the capture of different insect types.

In an attempt to address these questions, we studied the functional diversity of *Nepenthes* pitcher plants in a sample of species differing in their geographic origins and habitats. We measured the traits directly involved in the “slippery” and “viscoelastic” strategies, i.e. waxiness (quantity and density of wax coating the inner pitcher walls) and viscoelasticity of the digestive liquid. We used insect bioassays to compare the retentive ability of different *Nepenthes* species and measure how efficiently pitcher waxiness and fluid elasticity contribute to the retention of each type of prey. Finally, we investigated whether waxiness and viscoelasticity are inter-related.

Material and methods

Studied plants

One of the authors (J.-J. L.) owns a greenhouse collection of *Nepenthes* pitcher plants (located in Peyrusse-Massas, Gers, France) which in 1995 was recognized as the National French Conservatory of Carnivorous Plants. The insect bioassays performed in April 2007 employed the following subset of 12 *Nepenthes* species: *Nepenthes ampullaria, N. fusca, N. longifolia, N. maxima, N. mirabilis* var. *echinostoma, N. petiolata, N. rafflesiana* var. *typica, N. ramispina, N. spathulata, N. spectabilis, N. tobaica* and *N. ventricosa*. These species are representative of the ecological and geographical diversities found within the genus (terrestrial/epiphytic climbers; lowland/mountain species; species originating from Borneo, Sumatra, Sulawesi, Philippines and Peninsular Malaysia). One freshly opened lower pitcher (opening dating approximately one week previously) was selected on three different individuals of approximately the same size (30-60 cm) in each of the 12 species. A total of 36 pitchers were used.

In April 2008, a larger sample corresponding to 23 taxa and 21 species was used for comparisons of pitcher waxiness and viscoelasticity within the genus. This sample comprised all the previously cited species, except *N. ramispina* and *N. maxima* which did not produce any lower pitchers at the time of our study, together with 13 other taxa: *N. albomarginata, N. burgbidgeae, N. copelandii,* *N. eymae*, *N. glabrata*, *N. gracilis*, *N. macrophylla*, *N. madagascariensis*, *N. mindanaonensis*, *N. mirabilis* var. *typica*, *N. rafflesiana* var. *elongata*, *N. tenuis* and *N. vogelii*. Waxiness and viscoelasticity were measured for each species in a single pitcher that had been open for about a week.

Measurement of insect retention ability

Experiments comparing the trapping ability of the 12 *Nepenthes* species were carried out in April 2007 under homogeneous temperature and hygrometry conditions (26-27°C, 80-90%). Retention rates in the 12 species were compared for the ant *Lasius niger* (Hymenoptera, Formicidae, Formicinae) and the fly *Calliphora vomitoria* (Diptera, Calliphoridae). According to the prey spectra of *Nepenthes* species published so far (reviewed by Juniper *et al.* 1989; (Ellison & Gotelli, 2009), ants and flies are the most commonly trapped insects. Though the species used in our bioassays are not included, at least for the ants, in the natural prey fauna of the *Nepenthes* species tested, their size and shape are quite common in their order. Our aim here was simply to compare the ability of different *Nepenthes* species to retain flying insects versus crawling insects. Hence, any ants or flies could be used provided they did not exhibit any outstanding features, and this appeared to be a valid approach. A colony of *L. niger* found near the greenhouses provided us with worker ants. They were transferred to a plastic tube and placed on the pitcher rim, the so-called peristome (Fig. 1a). About 500 laboratory-bred *C. vomitoria* larvae were kept at 30°C for 7 days until adults emerged. The adults were then collected and confined to a glass jar connected to a cylindrical mesh, the aperture of which was closed by a piece of string. Once some of the flies emerged, the string was removed and the few emerging flies were collected in an inverted beaker connected to the mesh. Beaker and mesh were then disconnected from the jar, which was rapidly closed with a temporary cap, then “slipped” onto a pitcher, enclosing pitcher and flies together (Fig. 1b) by attaching the end of the mesh around the tendril sustaining the pitcher. These experimental set-ups allowed ants and flies direct access to the pitcher. Each trial consisted of an insect's fall into the pitcher and insect fate was recorded as the binary outcome: retained/not retained within the pitcher. We observed each insect until it died or escaped from the pitcher. An insect was considered as retained if it did not successfully escape from the pitcher. We considered three different pitchers for each of the 12 *Nepenthes* species, and performed 10 tests per pitcher, each time with a different ant and 10 tests, each time with a different fly, gathering a total of 720 binary outcomes and 72 retention rates.

Quantitative measurement of the characters involved in trapping

All the (intra + epicuticular) wax in the pitchers was extracted using warm chloroform as described by Riedel (Riedel *et al.*, 2003) and weighted to within 1 µg on a Sartorius MC5 balance (Gottingen, Switzerland – Fig. 1c). Because the plants are fragile, only one pitcher per species was used for wax extraction and measurement. The total weight of wax was used as an explanatory variable for retention rates: it takes account of not only the thickness but also the length of the waxy zone, and both parameters are probably important characteristics of the slippery trap. Each species' relative investment in ‘wax’ and ‘liquid viscoelasticity’ was determined by measuring the density of wax, estimated as the weight of wax per cm² of pitcher wall. This measurement of wax density is relevant in this case because the viscoelasticity measurement also reflects density, i.e. that of polymers in the fluid. In any case, the two different measurements of wax quantity plotted against fluid viscoelasticity showed similar statistical trends.

Fluid viscoelasticity was first estimated by observing the presence or absence of a filament when the fluid was stretched between two fingers. This qualitative measurement of viscoelasticity was performed on the fluid of each of the three pitchers in the 12 *Nepenthes* species studied in April 2007. Appropriate equipment for rheological studies was acquired in April 2008 and fluid from the 23 taxa was subject to a quantitative measurement of its viscoelasticity. An estimation of pitcher fluid viscoelasticity was obtained by measuring elastic relaxation time, defined by the time required for a filament of fluid to break, as described by Gaume and Forterre (2007). The fluid was subject to vertical strain by rapidly lifting a thin rod (diameter D_0 = 3.0 mm) 3cm vertically from a 40 µl sample of liquid, thus creating an elongated liquid filament. The subsequent thinning speed and time to filament rupture were recorded at a high spatial and temporal resolution (31 pixels/mm and up to 3500 frames/s) using a Phantom Miro IV high-speed camera (Vision Research, New Jersey, USA) and a Nikkor 60 mm macro lens (Nikon, Japan). The recordings were then analysed using an Image J - R script (Abramoff *et al.*, 2004, R Development Core Team, 2009), that we developed for this purpose (Fig. 1d). Each fluid was tested in triplicate and mean relaxation time calculated as an estimation of its viscoelasticity. The relaxation time of distilled water was measured six times as the non-viscoelastic reference fluid. All measurements were performed under homogeneous temperature conditions (25-26°C).

Statistical analyses

All statistical analyses were carried out using R software (R Development Core Team, 2009). Logistic regressions were used to explain the variability in insect retention success. Backward procedures were adopted for model selection, starting with removal of the non-significant, highest-order interactions

Two logistic models were used to address the following questions: i) does retention success vary between *Nepenthes* species, types of insect (ant/fly) and pitchers within a given species? and ii) could this variability be explained by the trapping features of the species, e.g. the waxiness of pitcher walls and the viscoelasticity of pitcher fluid? The first model was an ANOVA which set ‘species’, ‘pitcher’ nested within ‘species’, ‘insect type’ and the ‘species’ × ‘insect type’ interaction as explanatory factors for observed retention successes. As the pitcher effect was not significant, we then pooled retention success data for the three pitchers of each species and performed a second analysis that aimed to explain species retention rates (number of tests per species = 30) by their trapping features. Two ANCOVAs were thus performed on retention rates, one for each type of insect, with ‘presence/absence of a viscoelastic liquid’ and ‘quantity of wax in the trap’ as explanatory variables. Corrections for overdispersion were applied when necessary using the quasibinomial error distribution implemented in R.

Relaxation times for the 23 digestive fluids measured in April 2008 were compared by Student's t test with the capillary pinch-off time for water, i.e. the shortest breaking time for a filament. It should be noted that elastic relaxation times shorter than the capillary pinch-off time for water cannot be measured using this capillary break-up method (Rodd *et al.*, 2005). A fluid was conservatively qualified as viscoelastic if its relaxation time was significantly longer than the capillary pinch-off time for water, with $P < 0.01$. To determine whether the quantity of wax was inversely related to the viscoelasticity of the digestive liquids, we selected the species shown to have a viscoelastic fluid and tested whether the quantity of wax fitted a hyperbolic function of liquid viscoelasticity. Even if functional relation between traits should be considered in a phylogenetic framework (species traits are not statistically independants), phylogenies available (Meimberg *et al.*, 2006) are poorly resolved and species sample cover all the distribution of the genus which may reduce the phylogenetic bias.

Fisher’s exact test was performed on the contingency table (viscoelastic/non-viscoelastic liquid *vs.* mountain/lowland species) to compare statistically the frequencies of species associated with viscoelastic fluid in mountain and lowland species. The altitude of the studied species was obtained from different bibliographic sources (Clarke, 1997, Cheek & Jebb, 2001, Clarke, 2001, McPherson, 2009), and a threshold of 1000 m was applied to distinguish between lowland and mountain species.

Results

Functional diversity in the trapping system of Nepenthes

The species studied differed significantly in terms of their retention success for the insects employed in our bioassays (mean= 67 % ± 21 % of insects retained, significant species effect on retention success in the logistic regression model – Table 1, Fig. 2) and all were more efficient for ants than flies (significant insect effect, percentage of ants retained = 72 % ± 18 %, flies = 62 % ± 27 %, SD given in the text - Table 1). However, some species were more efficient for ants while others retained flies more easily (significant insect × species interaction – Table 1). No significant difference was noted for retention success between pitchers of the same species (pitcher (species): $\chi^2 = 19.73$, df = 24, $P = 0.71$).

The efficiency of pitcher wax and viscoelastic fluid is insect-dependent

The difference in insect retention efficiency between species can be explained by variations in pitcher waxiness. The total weight of wax averaged 1.85 mg and ranged from 0.28 mg in *N. ampullaria,* which has no visible epicuticular wax in its pitchers, to 3.40 mg in *N. maxima* which bears a thick layer of epicuticular wax in its pitchers. Fluid viscoelasticity also varied with species. The pitchers of six species (*Nepenthes ampullaria, N. longifolia, N. mirabilis, N. ramispina, N. spectabilis* and *N. ventricosa*) were shown to contain a non-viscoelastic, apparently water-like fluid whereas the pitchers of six other species (*N. fusca, N. maxima, N. petiolata, N. rafflesiana, N. spathulata* and *N. tobaica*) contained a fluid shown to be viscoelastic by the creation of a filament when stretched between two fingers. The highest retention rates were observed in species with viscoelastic fluids, and the lowest were observed in species with water-like fluids (Fig. 2).

Differences in wax quantity and fluid viscoelasticity explained most of the variations observed in the trapping ability of the species which also differed with regard to insect type. For ants, retention rates increased significantly with wax quantity and fluid viscoelasticity (covariance analysis, Table 2a, Fig. 3a). For flies, retention rates did not significantly depend on the amount of wax but on fluid rheometry as rates were far higher when the fluid was viscoelastic (Table 2b, Fig. 3b). Our observation of insect behavior corroborated these results. Ants that fell into the liquid close to the pitcher wall were not extensively wetted and were often observed to reach and climb up the pitcher wall but then slip frequently when reaching the waxy zone. By contrast, flies that were not extensively wetted by the liquid were sometimes observed to climb up the pitcher wall and successfully take off from there, occasionally without even touching the waxy layer. If wetted by the viscoelastic liquid, the insects had little chance of escaping from it. The more they struggled in the fluid, the greater it resisted their movements and they rapidly became exhausted and drowned.

Relative investments in wax and liquid viscoelasticity

Wax quantities measured in April 2008 were consistent with those obtained in 2007. Wax densities ranged from 0.022 mg.cm^{-2} (*N. ampullaria,* wax quantity = 0.229 mg) to 0.608 mg. cm^{-2} (*N. macrophylla,* wax quantity = 3.180 mg) averaging 0.124 mg.cm^{-2} (median = 0.087 mg.cm^{-2}, SD = 0.135). In all, 15 of the 23 species were viscoelastic, i.e. their relaxation time was significantly longer than that of water, the non-viscoelastic reference fluid (t test results in Table 3). *N. longifolia* and *N. spectabilis* appeared to be slightly viscoelastic but were not classified as viscoelastic in 2007, perhaps because their viscoelastic filaments were not visible by direct observation. Of the 13 mountain species, 12 were found to be viscoelastic whereas only three lowland taxa (two species, *N. longifolia, N. rafflesiana* var. *typica* and *N. rafflesiana* var. *elongata*) were found to be viscoelastic of the 10 lowland taxa (nine species) in our sample. These proportions differed significantly (Fisher's exact test performed on the 23 taxa: $P = 0.006$; on the 21 species: $P = 0.003$) demonstrating that mountain species more often possess viscoelastic fluids. By way of a comparison, the values obtained for water ranged from 0.024 s to 0.028 s (N = 6). None of the species with very waxy pitchers was found to also possess a very viscoelastic fluid, and *vice-versa*. Moreover, the quantity of wax produced in the pitchers of viscoelastic species, and the viscoelasticity of their fluids, were seen to be inversely related: the more wax a species produces, the less viscoelastic its fluid (wax density ($mg.cm^{-2}$) = 0.013 + 0.014/ relaxation time (s), $F_{1,12} = 14.19$, $P = 0.001$, $R^2 = 0.75$ – Fig. 4).

Discussion

Our comparative study of the trapping systems of *Nepenthes* pitcher plants generated three important results. Firstly, and contrary to common belief (but see Gaume & Di Giusto 2009; Bonhomme *et al.* 2010), the different species show functional diversity in their retentive devices and do not rely solely on the slipperiness of their trap to capture insects. The results of this study show that *Nepenthes rafflesiana* is not the only species to possess a viscoelastic fluid. This character may be widespread within the genus as found in two thirds of the species in our study. Secondly, investments in the "waxy" trait and in "viscoelastic fluid" appear, each one, to be made at the expense of the other. This seems to define two different trapping strategies in these pitcher plants, a "slippery" strategy and a more "viscoelastic" strategy. Thirdly, these two trapping devices do not target the same type of prey, and *Nepenthes* species with one or the other of the trapping strategies do not usually occur in the same natural habitat. Wax appears to be efficient only for ants whereas viscoelasticity proved to be a powerful trapping device for both insect types and is more often found in mountain than in lowland species.

A widely-shared viscoelastic trap in Nepenthes pitcher plants and the question of its origin

The fluid contained in the pitchers of most of the *Nepenthes* species studied was viscoelastic, and this might therefore represent the rule in the genus rather than an exception. Contrary to wax, whose efficiency as a trapping device has been shown to be quantity-dependent, even low viscoelasticity appears to contribute to a plant's trapping ability. Therefore, it is surprising to note that some fluids in *Nepenthes* pitchers are highly viscoelastic. It is possible that this viscoelasticity increases with plant age (Gaume & Di Giusto, 2009a) or with pitcher age (Bauer, Willmes & Federle 2009). But, the plants and pitchers of the different species used in our study were carefully chosen to be of similar age. Another explanation could be that, as the plant species differ in their habitats, some are more subject than others to rainfall and humidity (e.g. those of altitudinal mossy forests) and to subsequent fluid dilution by water. Greater production of the polymers that are assumed to cause the viscoelasticity of pitcher fluid might have been selected in some species, helping them cope with the problem of daily dilution. This hypothesis is corroborated by the results of Gaume and Forterre who showed that the elastic fluid of *N. rafflesiana* (which here appears to be among the most viscoelastic species), when diluted by 95% of water, was still sufficient to capture all the insects dropped into the pitchers (Gaume & Forterre, 2007).

Since relaxation times of less than 100 ms are impossible to detect without the use of a high-speed camera, viscoelastic fluids have probably gone unnoticed in many species and may be far more common than suspected. This raises the question of whether the viscoelastic fluids in *Nepenthes* species have a common origin. Interestingly, the glue secreted by the leaves of *Drosera*, another carnivorous genus, is composed of acid polysaccharides (Gowda *et al.*, 1982) and these have been demonstrated to be viscoelastic (Erni *et al.* 2008). This is also probably the case for the glue secreted by *Drosophyllum* (unpublished results). Both of these genera are the closest to *Nepenthes* in the phylogeny of the Caryophyllales (Heubl *et al.*, 2006) and their traps are known to function like flypaper (Juniper *et al.* 1989). We can therefore put forward the hypothesis that the viscoelastic, polysaccharide fluids in *Nepenthes* and in these other carnivorous genera have a common and thus plesiomorphic origin, with the glue of the other carnivorous genera simply containing a far higher concentration of polysaccharides than the fluid in *Nepenthes*.

Investment trade-off between trapping devices

The appearance of botanical carnivory and the evolution of specialised traps are subject to powerful cost-benefit constraints (Givnish *et al.*, 1984, Ellison & Gotelli, 2001, Pavlovic *et al.*, 2007). We can therefore assume that it is costly for carnivorous plants to produce modified leaves with lower photosynthetic capacities (Pavlovic *et al.*, 2007, Pavlovič *et al.*, 2009), and that the biosynthesis of trapping features is subject to selective pressure and can be maintained throughout evolution only if the cost of these features is exceeded by the benefits they provide in terms of insect-derived nutrients. Development of the waxy zone, mainly composed of aliphatic compounds dominated by very long chain aldehydes (e.g. triacontanal or dotriacontanal containing 30 or 32 carbon atoms, respectively (Riedel *et al.*, 2003, Riedel *et al.*, 2007)), is metabolically costly for the plant. The molecules responsible for digestive fluid viscoelasticity are assumed to be long-chain polysaccharides (Gaume & Forterre, 2007) that must also be costly to synthesise. This may explain why none of the species we tested possesses both very viscoelastic fluids and very waxy pitchers. The inverse relationship that links these two quantitative traits might illustrate the existence in the plant of an investment trade-off.

Interestingly, a few plant species in our study showed both non-viscoelastic fluids and only slightly waxy pitchers, or pitchers that contained no epicuticular wax at all, such as *N. ampullaria* and *N. ventricosa.* These plants are outliers in the *Nepenthes* genus. Maybe the pitchers do not have a strictly carnivorous diet; this is the case for *N. ampullaria* which obtains part of its nitrogen from leaf debris (Moran *et al.*, 2003). An alternative explanation is that they utilise other trapping strategies. *N. ampullaria,* maybe relies uniquely on its peristome that forms a steep slop, to trap its prey. Several other features such as water-dependent structures facilitating insect-aquaplaning (Bohn & Federle 2004; Bauer, Willmes & Federle 2008) or specific pitcher morphology, might favour both insect capture and retention. But, the pseudo R^2 estimated for the logistic regressions indicated that wax and fluid viscoelasticity explained at least half for ants and three quarters for flies of the variability observed in the trapping success in the *Nepenthes* species studied. The question is therefore raised as to which selective factors favour the waxy or the viscoelastic strategy?

The role of prey in the evolution of different trapping strategies

Pitcher wax causes insects to slide (Juniper & Burras, 1962, Gaume *et al.*, 2002) and viscoelastic fluid acts to retain them (Gaume & Forterre, 2007). And the results obtained in our study show that the efficiency of these strategies is prey-dependent. Wax is more efficient on ants than on flies whereas viscoelasticity is very efficient on both insect types and definitely more efficient than wax on flies. Winged insects are able to take off from the pitcher wall without even touching the waxy surface, and even if they do enter into contact with it, the wax acts only on their attachment systems (Gaume *et al.*, 2004, Gorb *et al.*, 2005) not their flying system. By contrast, crawling insects have no other option than to cope with the wax that contaminates their pads and causes them to lose adhesion. Moreover, since winged insects have a higher surface/volume ratio than crawling insects, they offer a larger surface area for the viscoelastic fluid to exert its retentive force and this may explains why they are more often retained in *Nepenthes* liquids (Gaume *et al.*, 2002, Gaume & Forterre, 2007).

If the capture efficiency of wax and viscoelastic fluid are insect-dependent, then local entomofauna may exert marked selective pressure for the development of a given trapping strategy. For any given pitcher waxiness, a “viscoelastic strategy” is needed to trap flies with the same efficiency as ants. This means that habitats dominated by ants, such as the lowland forests of Borneo (Gunsalam, 1999, Davidson *et al.*, 2003) may favour the development of a waxy “slippery” strategy. On the other hand, habitats dominated by flying insects may favour the development of a “sticky”, viscoelasticity-based strategy. Such habitats are generally found at higher altitudes where ants are few in number but flying insects relatively more abundant (Collins, 1980). This can also temporarily be the case for lowland, open, and regularly flooded habitats such as those inhabited by *Nepenthes rafflesiana* var. *typica* (Gaume (Gaume & Di Giusto, 2009a) which is associated with a flower scent cue that more specifically targets flying insects (Di Giusto *et al.* 2010).

We therefore put forward the hypothesis that the scarcity of ants in tropical mountains (Borneo (Collins, 1980, Clarke *et al.*, 2009)], Philippines (Samson *et al.*, 1997) and the relative abundance of flying insects (Collins, 1980) provide part of the explanation for the widespread viscoelastic strategy among mountain *Nepenthes* species. A comparative study (Adam, 1997) corroborated this hypothesis by showing that mountain species tend to trap a larger prey spectrum including more dipterans and coleopterans than lowland species, which were recorded to trap mostly ants. Furthermore, at least seven species in mountain mossy forests, and known to possess a highly viscous fluid, are reported to be specialised in the capture of flying insects, i.e. *N. inermis* reported to be (under the name of *N. bongso*) specialised in trapping midges (Kato, 1993), *N. aristolochioides*, specialised in trapping midges, *N. dubia, N. jamban*, *N. eymae* & *N. talagensis* specialised in trapping small dipterans (McPherson 2006) and *N. jacquelinae* observed to trap mainly larger flying prey (Clarke, 2001). Interestingly, the pitchers of such species do not contain a waxy zone and are all funnel-shaped.

Prey is central to the ecology of carnivorous plants yet few comparative studies have been conducted on the prey spectra of *Nepenthes* species (but see Kato *et al.* 1993; Adam 1997). Further studies to compare the prey spectra of *Nepenthes* species with the entomofauna found in their habitats, and relating this to their insect-trapping devices, would help understand the ecological mechanisms underlying the evolution and diversification of these pitcher plants.

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Tables

Variability in insect retention

Response variable: retention success	**df**	χ^2	**P(>** χ^2**)**
Covariate			
Species	11	91.22	< 0.0001
Insect	1	8.8	0.003
Species × Insect	11	46.24	< 0.0001

Table 1: Results of logistic model testing for *Nepenthes* species and insect type effects on trapping success

(a) Ant retention success dependent on viscoelasticity and wax quantity, Cox-Snell pseudo $R^2 = 0.53$

Response variable: retention rates	**df**	χ^2	**P(>** χ^2**)**
Covariate			
Viscoelasticity	1	8.1	0.004
Wax quantity	1	5.35	0.021

(b) Fly retention success dependent on viscoelasticity, Cox-Snell pseudo $R^2 = 0.74$

Response variable: retention rates	**df**	χ^2	**P(>** χ^2**)**
Covariate			
Viscoelasticity	1	25.54	< 0.0001
Wax quantity	1	2.58	0.108

Table 2: Results of logistic regression testing for wax quantity and fluid viscoelasticity effects on trapping success for ants (a) and flies (b).

***Nepenthes* species**	**Geographic origin**	**Altitude**	**Mean relaxation time (s)**	**P (>t)**
Albomarginata	B, M, S	lowland	0.027 ± 0.152	0.152
Ampullaria	B, M, NG, S, T	lowland	0.025 ± 0.532	0.532
Gracilis	B, M, Sul.	lowland	0.024 ± 0.835	0.835
Longifolia	S	lowland	0.035 ± 0.000	< **0.001**
Madagascariensis	Madagascar	lowland	0.025 ± 0.800	0.800
Mindanaonensis	P	lowland	0.022 ± 0.999	0.999
Mirabilis var. *echinostoma*	B	lowland	0.028 ± 0.169	0.169
Mirabilis var. *typica*	SE-Asia	lowland	0.026 ± 0.363	0.363
Rafflesiana* var. *elongata	B	lowland	0.533 ± 0.001	< **0.001**
Rafflesiana* var. *typica	B, M, S	lowland	0.601 ± 0.000	< **0.001**
Burgbidgeae	B	mountain	0.635 ± 0.001	**0.001**
Copelandii	P	mountain	0.465 ± 0.001	< **0.001**
Eymae	Sul.	mountain	0.096 ± 0.000	< **0.001**
Fusca	B	mountain	1.642 ± 0.003	**0.003**
Glabrata	Sul.	mountain	0.085 ± 0.000	< **0.001**
macrophylla	B	mountain	0.036 ± 0.000	< **0.001**
Petiolata	P	mountain	0.123 ± 0.006	**0.006**
Spathulata	S	mountain	0.338 ± 0.001	< **0.001**
Spectabilis	S	mountain	0.413 ± 0.003	**0.003**
Tenuis	S	mountain	0.753 ± 0.000	< **0.001**
Tobaica	S	mountain	0.241 ± 0.000	< **0.001**
Ventricosa	P	mountain	0.025 ± 0.800	0.800
Vogelii	B	mountain	0.052 ± 0.007	**0.007**

Table 3: Measurements of fluid viscoelasticity for each studied species. Geographic origin (B = Borneo, M = Peninsular Malaysia, N.-G = New-Guinea, P = Philippines, Sul. = Sulawesi, S = Sumatra) and type of habitat (lowland/mountain) is provided for each species. Student's *t* test was used for each species to determine whether digestive fluid relaxation times significantly differ from those of distilled water, and the corresponding P-value is provided. “Viscoelastic species” are boldfaced and are more frequent among mountain species than lowland species, as confirmed by Fisher's exact test.

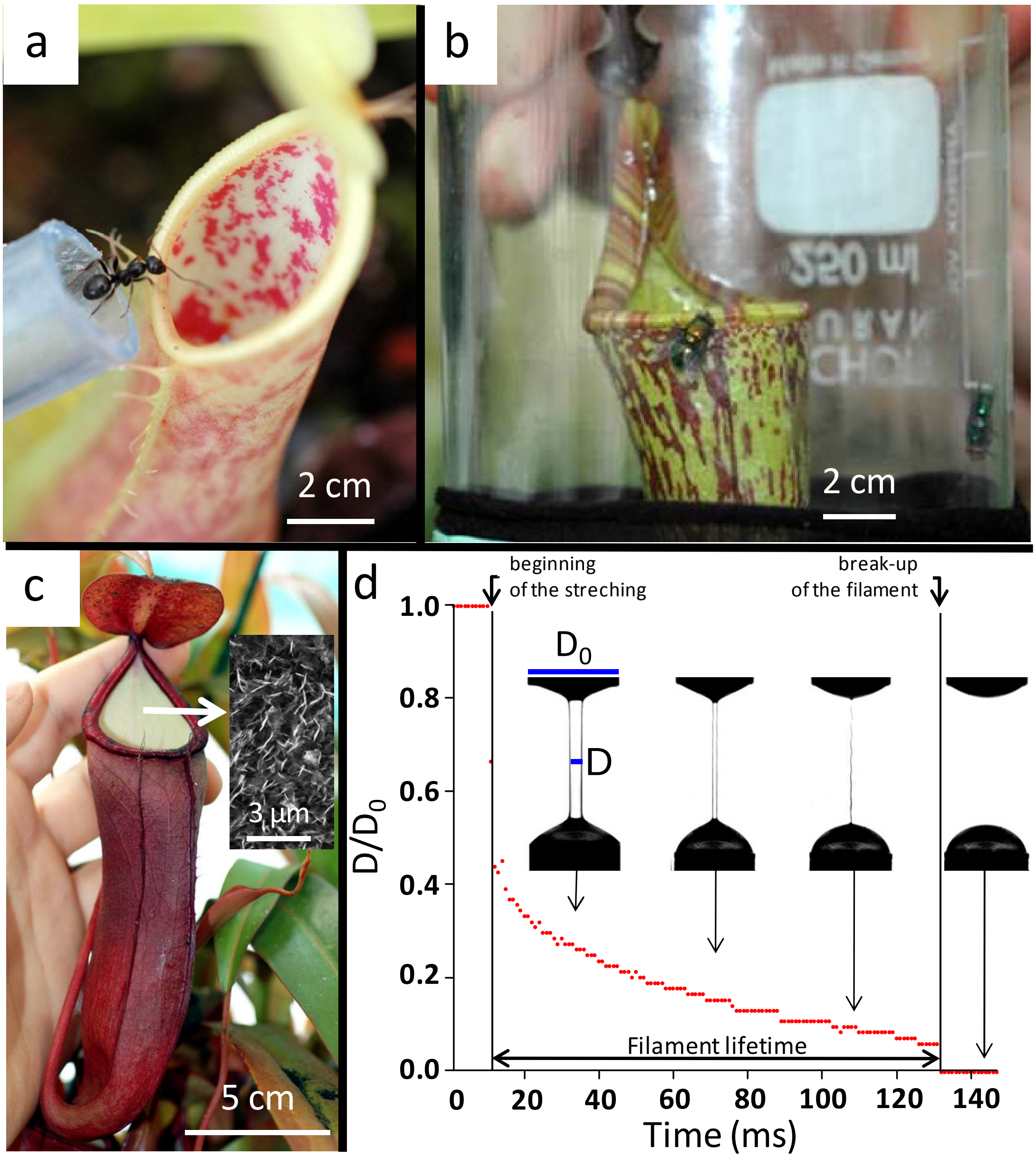

Figure 1: Experimental designs used to test the effects of pitcher waxiness and fluid viscoelasticity on ants and flies. (a) Ants were handled using a soft tube and allowed to walk freely on the pitcher rim. (b) The jar containing the experimental flies was opened and linked by a gauze mesh to a glass beaker covering the upper part of the pitcher. (c) The photograph shows a *Nepenthes* pitcher with a waxy zone (pale area, arrow) from which crystalline wax (shown in insert by a Scanning Electron Microscope view) was extracted using hot chloroform. (d) The extensional rheometry measurements of the digestive fluid were made by high-speed video-recording and analyses of the thinning dynamics of a filament (measure of its diameter D related to initial diameter D_0) created by vertically stretching a droplet of digestive fluid between two plots distant from 3 cm apart. Filament lifespan was used to estimate fluid viscoelasticity.

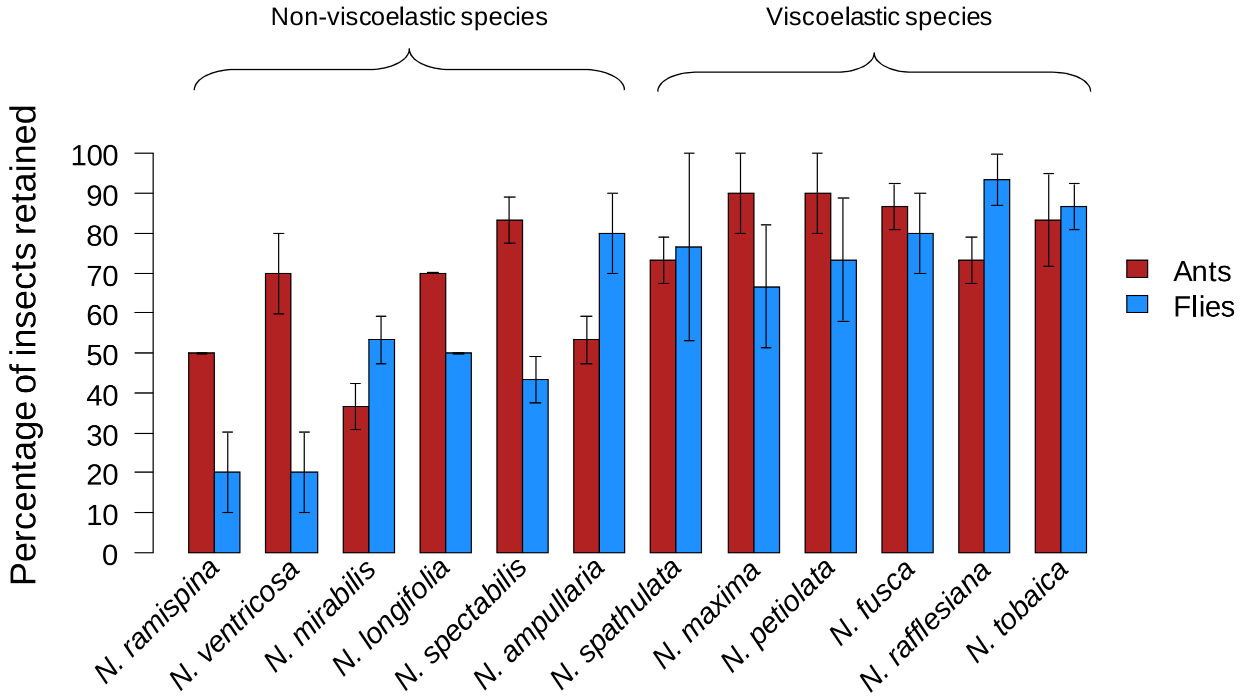

Figure 2: Percentage of ants and flies retained inside the pitchers of 12 *Nepenthes* species.

Species are ranked for increasing mean capture rate. Error bars refer to SE.

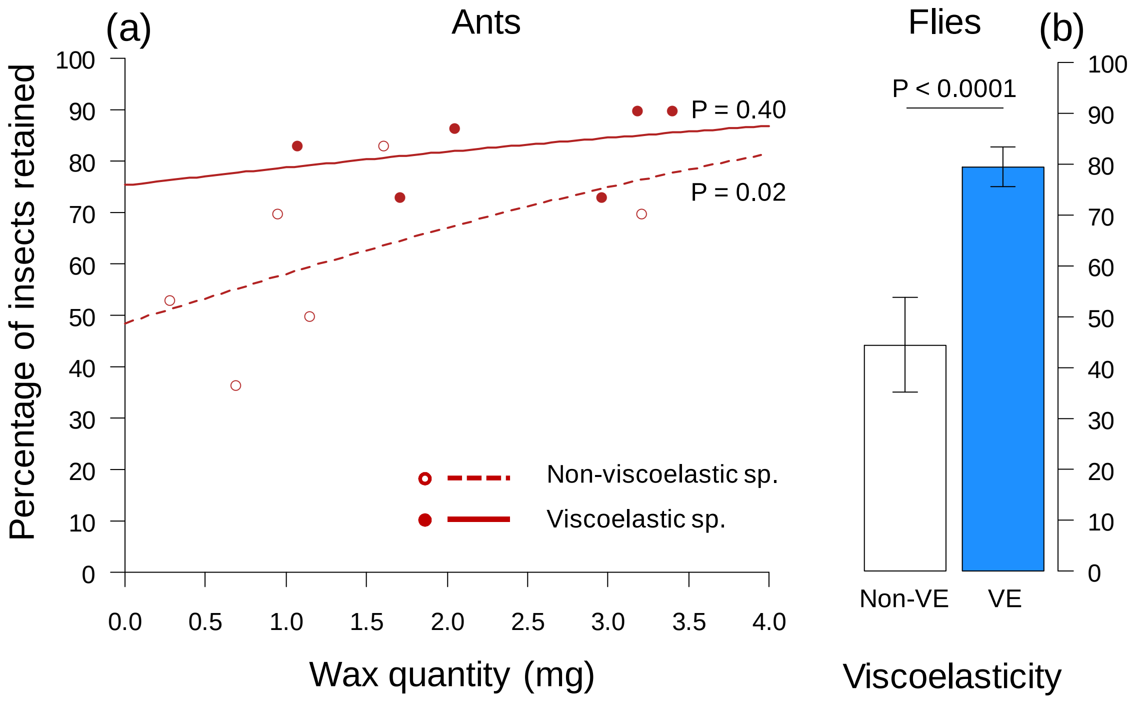

Figure 3: (a) As shown in Table 2a, the percentage of ants retained in the trap increases with both fluid viscoelasticity and wax quantity. The estimated trend curves shown here were obtained from estimated parameters in the two different logistic models employed on “non-viscoelastic species” (χ^2=5.08, P = 0.02) and “viscoelastic species” (χ^2 = 0.70, P = 0.40). (b) For the flies, retention rates depended only on fluid viscoelasticity and were shown to be higher for species associated with viscoelastic fluids than for species associated with water-like fluids.

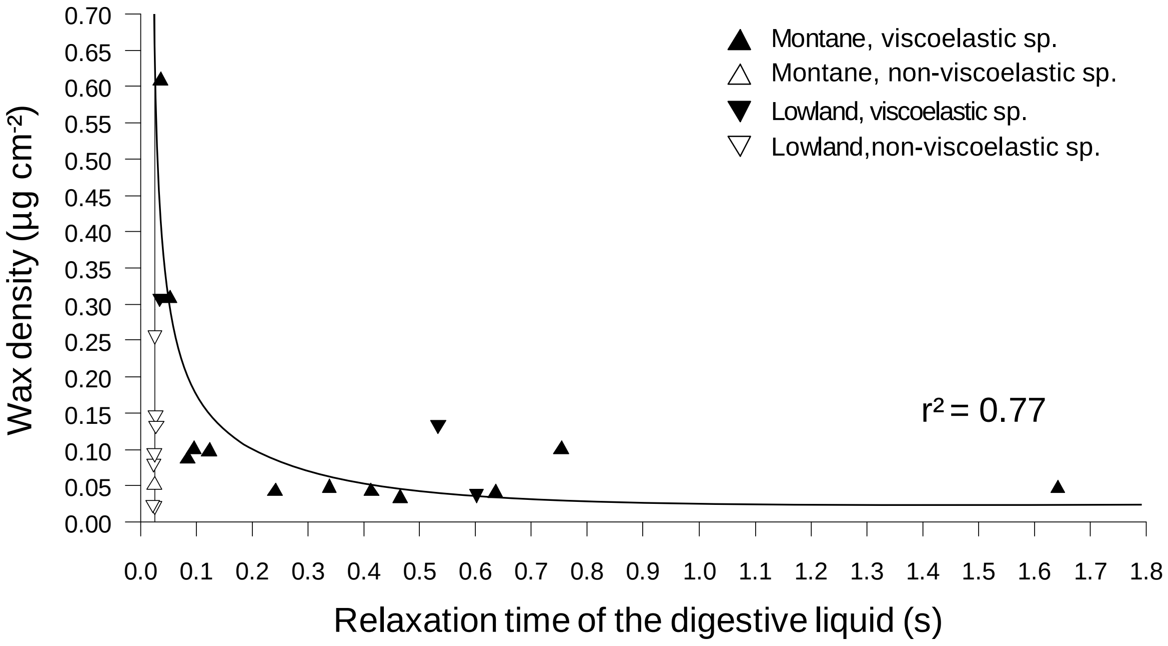

Figure 4. Plant investment trade-off for the two trapping devices (pitcher waxiness and fluid viscoelasticity). The two trapping devices are inversely related. Most mountain taxa contain a viscoelastic fluid while most lowland taxa use a water-like fluid (Table 3). The vertical line represents the upper value of the relaxation time obtained for water.

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Chapitre I.1

UNE STRATÉGIE DE PIÉGEAGE ALTERNATIVE, LE CAS DU MUTUALISME DE *NEPENTHES BICALCARATA* ET *CAMPONOTUS SCHMITZI*

La nature de la relation qu'entretient la fourmi *Camponotus schmitzi* avec sa plante-hôte *Nepenthes bicalcarata* n'est pas clairement élucidée. Cette interaction intrigante entre une plante carnivore et une fourmi symbiotique suscite depuis plus d'un siècle de nombreuses hypothèses quant aux bénéfices réciproques des deux partenaires. *Nepenthes bicalcarata* héberge dans des domaties gonflées et creuses une espèce de fourmi qui y niche, élève son couvain et se nourrit de son nectar. Deux types de bénéfices pour la plante ont déjà été proposés : (1) la protection contre la putréfaction des urnes par le retrait sélectif des plus grosses proies du liquide par les *C. schmitzi* et (2) une défense spécifique contre des charançons qui s'attaquent aux urnes en formation. La plupart des fourmis mutualistes fournissant une protection aux feuilles de leur plante-hôte contre les herbivores, cette hypothèse pourrait être soulevée pour le cas de *Camponotus schmitzi* mais la présence de cette fourmi sur les feuilles et l'extérieur de l'urne pourrait cependant dissuader les insectes et diminuer le nombre de visiteurs, diminuer le nombre de proies capturées par *Nepenthes bicalcarata* et donc sa fitness. Dans ce cas, les structures développées par la plante devraient être contre-sélectionnées. **Le Manuscrit I.2** apporte un éclairage nouveau à ce mutualisme et explore l'hypothèse que la fourmi puisse contribuer à la nutrition de la plante, via la capture et la digestion d'insectes.

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THE PLANT-ANT *CAMPONOTUS SCHMITZI* HELPS ITS CARNIVOROUS HOST-PLANT *NEPENTHES BICALCARATA* TO CATCH ITS PREY

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Abstract

The Bornean climber, *Nepenthes bicalcarata*, is unique among plants because it is both carnivorous and myrmecophytic, bearing pitcher-shaped leaves and the ant *Camponotus schmitzi* within tendrils. We explored, in the peatswamp forests of Brunei, the hypothesis that these ants contribute to plant nutrition. We first tested whether ants increased plant's capture rate. We found that unlike most plant-ants, *C. schmitzi* do not exhibit dissuasive leaf-patrolling behaviour (zero patrol on 67 pitchers of 10 plants) but lie concealed under pitcher rim (13 ± 6 ants per pitcher) allowing numerous insect visits. However, 47 out of 50 individuals of the largest visitor dropped into the pitchers of five plants were attacked by ants and the capture rate of the same but ant-deprived pitchers decreased three-fold. Therefore, hunting by ambush, these ants help the plant to catch prey. We then tested whether ants participated in plant's digestion. We showed in a 15-d-long experiment that ants fed on prey and returned it in pieces in seven out of eight pitchers. The 40 prey deposited in ant-deprived pitchers remained intact indicating a weak digestive power of the fluid, confirmed to be only weakly acidic (pH ~5, n = 67). The analysis of 10 pitcher contents revealed that prey, mainly ants and termites, was very numerous (~ 400 per pitcher per plant) and highly fragmented. Altogether, these data suggest a positive effect of *C. schmitzi* on both prey intake and breakdown. This ant-plant interaction could thus be a nutritional mutualism involving the unusual association of carnivory and myrmecotrophy.

Keywords: Ant-plant mutualism, myrmecophyte, myrmecotrophy, pitcher plant, predation strategy

Introduction

Scarcity of essential nutrients has led to the evolution of alternative strategies of nutrition in plants, such as myrmecotrophy and carnivory, which allow them to obtain nutrients from animals (Juniper *et al.* 1989, Thompson 1981). Myrmecotrophy refers to ant-fed plants (Beattie 1989, Solano & Dejean 2004). Most myrmecotrophic plants are also myrmecophytes, i.e. plants that harbour ants in specialized cavities called domatia (McKey *et al.* 2005). These plants, often epiphytes, assimilate the decomposition products of the ant faeces and debris accumulated in modified stems, rhizomes or leaves (Huxley 1978, Janzen 1974, Treseder *et al.* 1995).

Carnivorous plants derive some key nutrients, such as nitrogen, from arthropods that they capture and digest in their traps (Ellison & Gotelli 2001, Juniper *et al.* 1989). The carnivorous genus *Nepenthes* comprises more than 100 species (Clarke 1997, McPherson 2009), mainly distributed in South-East Asia. Its traps are leaves modified as pitchers filled with an enzymatic fluid and a community of mostly dipteran and bacterial 'infauna' involved in the digestion process (Beaver 1983, Clarke & Kitching 1993, Creswell 2000). The pitchers exhibit various combinations of characters involved in insect attraction and capture, such as UV patterns (Moran 1996) and sweet odours (Di Giusto *et al.* 2010, Moran 1996), wettable rims (Bauer *et al.* 2009, Bohn & Federle 2004), slippery waxy surfaces (Gaume & Di Giusto 2009, Gaume *et al.* 2002, Juniper *et al.* 1989) and viscoelastic digestive liquid (Di Giusto *et al*. 2008, Gaume & Forterre 2007). A few *Nepenthes* species have been shown to display unusual N-sequestration strategies, obtaining nitrogen from plant debris (Moran *et al.* 2003) or vertebrate faeces (Clarke *et al.* 2009).

Nepenthes bicalcarata Hook. f., endemic to the peat-swamp forests of northern Borneo, is the only *Nepenthes* species known to be myrmecophyte: it harbours in its tendrils the species-specific ant *Camponotus schmitzi* Stärke. In this study we explore the hypothesis that *N. bicalcarata* uses both carnivory and myrmecotrophy to circumvent nutrient scarcity.

This ant-plant association still remains intriguing. It was first proposed to be a mutualism in which the ants gain nectar and nest sites (Figure 1a-c) from their host-plant and confer on it some protection against pitcher putrefaction (Clarke & Kitching 1995). The authors reported that *C. schmitzi* ants were unaggressive but able to safely swim into the digestive liquid and remove large dead prey items, which paradoxically benefits the plant in avoiding sudden ammonium releases and subsequent pitcher putrefaction. In a later study, these ants were shown to aggressively defend their host-plant against a specific weevil that feeds on pitcher buds (Merbach *et al.* 2007).

These apparently two contradictory observations on the aggressiveness of *Camponotus schmitzi* towards insects raises the fundamental question of how these ants interact with insects visiting the mature and open pitchers for their nectar, hereafter called nectar visitors. Do they dissuade, as do most plant-ants, these visitors which are also potential prey of the pitcher plant or do they conversely facilitate their capture? Another not completely resolved question concerns their feeding behaviour and its impact on the plant’s intake. To what extent do these ants consume the prey inside the pitcher and could they play a role in its breakdown and digestion by the plant?

The answers to these questions might help unravel the nature of the interaction between *C. schmitzi* and its host-plant and explore the hypothesis that the carnivorous plant obtains a nutritional benefit from its ant symbiont. To this end, we first tested the hypothesis that the ants hunt and help the plant to catch its prey, by quantifying the patrolling behaviour and aggressiveness of *C. schmitzi* towards pitcher visitors and fallen prey and by measuring their impact on prey capture using an ant-exclusion experiment. We then tested the hypothesis that they regularly consume part of the prey and help in the plant’s digestion. Using a 15-d long prey-deposition experiment, we studied the frequency of their feeding behaviour towards experimental prey of two size-categories and its impact on prey breakdown. We also analyzed the prey contents of pitchers and measured the pH of the fluid, to obtain data respectively on the plant’s prey intake and on its digestive ability.

Study species and study site

Nepenthes bicalcarata is a liana that climbs up to 20 m and has enormous leaves with pitchers that are long-lived in comparison to other *Nepenthes* species (Cheek & Jebb 2001, Clarke 1997). The tendril that sustains its trap is swollen (Figure 1a) and inhabited by the small ant *Camponotus schmitzi* (Formicinae), that has never been reported living outside its host-plant (Clarke & Kitching 1995, Jolivet 1986). The most characteristic structures of *N. bicalcarata* are the two giant nectaries shaped like thorns that overhang the pitcher’s mouth (Figure 1b) and are exploited by *C. schmitzi* and other ants (Merbach *et al.* 1999).

All experiments were carried out in July-August 2009 in a mixed peat swamp and heath forest (4°44’N, 114°35’E) of Brunei Darussalam (northern Borneo) on *N. bicalcarata* upper pitchers that were all occupied by *C. schmitzi*.

Methods

Measure of ant territoriality and pitcher fluid acidity

To assess the territoriality and patrolling behaviour of *C. schmitzi* outside the pitchers, we counted over 1 min the total number of *C. schmitzi* workers and arthropod visitors on nectariferous external parts of the pitchers on a total of 67 functional pitchers of different age classes belonging to 10 plants.

All observations were made around 10h00 in sunny conditions over 10 d, i.e. in rather homogenous conditions of nectar production and insect activity. After each observation, the pH of pitcher fluid was measured using pH-indicator strips (Acilit® pH 0-6.0, Merck Chemicals, Darmstadt, Germany). We wished to examine on a large sample, representative of the different age-classes of pitchers, if the pH of this fluid was not very acidic as already observed by Clarke & Kitching (1993) and if such a low acidity was maintained throughout the pitcher life span. Constant neutral pH or moderate acidity would mean that the fluid is inoffensive towards the swimming ant symbiont but also not very efficient in dissolving the prey.

Insect retention experiment

We then tested the aggressiveness of *C. schmitzi* towards other insects inside the pitchers and tested their effect on prey retention. We selected, as experimental prey, ants commonly found feeding on the extrafloral nectaries of *N. bicalcarata*. We first tried to use a small-bodied ant species (*Crematogaster* sp. 1, total length = 3.5 mm, Table 1), one of the two most common visitors and prey of *N. bicalcarata,* but workers of this species were never observed to escape from the digestive liquid even when the pitchers were deprived of their *C. schmitzi* ants. Hence they could not be good candidates to test whether *C. schmitzi* deployed aggressiveness toward fallen prey. We thus chose *Polyrhachis pruinosa* Mayr, another common visitor and prey item of the plant (total length = 1.2 cm, Table 1, Figure 1b), as they were easier to handle and they usually succeeded in escaping from the liquid.

Five *C. schmitzi*-occupied pitchers belonging to five different plants were haphazardly selected. A *Polyrhachis* ant was dropped into the digestive liquid of each pitcher, and the fate (retained/escaped) and behaviour (time needed to escape from the pitcher, the number of times the ant fell back into the liquid) of this ant were observed and sometimes video-recorded. To drop the ant into the pitcher's digestive liquid, we first drew it into a soft tube and then blew it onto the digestive liquid without direct manipulation. We repeated this experiment with 10 different ants for each pitcher. Intervals between successive trials were less than 5 min when the ant succeeded in escaping within the 5 min. When the ant did not escape within 5 min, we observed it for more than 20 min, to check that it was effectively killed. The ant was then removed from the pitcher before the next trial. The results of this experiment were subsequently compared with the fate and behaviour of 10 *Polyrhachis* ants on the same pitchers but deprived of *C. schmitzi*. We used smoke produced by burning dead leaves to deter the latter. 20 trials were thus conducted per pitcher (10 trials with and 10 trials without *C. schmitzi*). An ant was considered as retained if it did not escape within 5 min. The ants that did not successfully escape within 5 min were observed to die, except for two ants that were observed to be exhausted, exhibiting very slow movements.

Data were analysed using the software package SAS v.9.1. We tested for an effect of *C. schmitzi* (fixed factor) and pitcher (random factor) on the percentage of ants trapped using a mixed logistic regression by using the macro GLIMMIX with a binomial error distribution. We tested for an effect of *C. schmitzi* (fixed factor) and pitcher (random factor) on the number of times the experimental ant slid back into the digestive fluid after escape attempt with a mixed Poisson regression model by using the macro GLIMMIX with a Poisson error distribution. Correction for over-dispersion was applied using the square root of the ratio of Pearson's χ^2 to the associated number of degrees of freedom. We tested for an effect of *C. schmitzi* (fixed factor) and pitcher (random factor) on the time required to escape, by using a mixed-model analysis of variance with the GLM procedure. For model selection backward procedures were adopted, starting with the removal of non-significant interactions.

Feeding behaviour of Camponotus schmitzi

A second experiment investigated the feeding behaviour of *C. schmitzi* towards deposited prey of two different sizes and comparable to the sizes of the prey we observed inside the pitchers of *N. bicalcarata*. Ten upper pitchers were haphazardly selected, each belonging to a different plant. Their arthropod contents were removed by filtering the liquid with a mesh and the digestive liquid was then returned to the pitchers. Tanglefoot© insect glue was applied on the leaves bearing the pitchers and on the vegetation in contact with the tendril so that no crawling insects other than *C. schmitzi* could reach the prey items. In each of the 10 arthropod-free pitchers, we introduced 10 entire bodies of a *Crematogaster* species and 10 gasters (abdomens) of *Polyrhachis pruinosa*. Neither of these prey items could be mistaken for any other insect that could have fallen - despite our precautions - into the pitchers. Firstly, the *Crematogaster* species used in this experiment comes from another site and was never observed in the site where the experiment was conducted. Secondly, we only used gasters of *Polyrhachis pruinosa* to be sure that they belong to our experimental prey items and not to the bodies of possibly newly fallen workers of this species, common in this site. The two prey items measured respectively 2 mm and 5 mm, both within the main size range of prey of *Nepenthes bicalcarata*. *Camponotus schmitzi* ants were removed from two of these 10 pitchers, as well as from their associated domatium. These two pitchers were used as controls to check that in the absence of *C. schmitzi*, no prey was removed from pitchers. Fifteen days later, the content of each of these 10 pitchers was collected, counted and observed using a binocular microscope. As the total numbers of experimental prey items were equal (= 10) for each pitcher and each prey category, we tested for an effect of prey category (fixed factor) and pitcher (random factor) on the number of items presenting obvious signs of ant predation by using a mixed Poisson regression model with the macro GLIMMIX specifying a Poisson error distribution.

Analysis of prey contents

The prey contents of 10 old but still functional pitchers (the pitcher borne by the node immediately below being senescent), all inhabited by *Camponotus schmitzi* and belonging to different individuals were collected in the same site in September 2008 in 10 vials filled with 70% alcohol, then analysed and counted in the laboratory using a binocular microscope. The necromass was composed of plant and animal parts as well as of small 'pellets' of fine particles that might be the faeces of *C. schmitzi* or of mosquito larvae, although we never observed such kinds of faeces in the pitcher fluid of other *Nepenthes* species that all bore mosquito larvae. Only the animal parts were considered in this analysis. The prey items found in these pitchers were highly disintegrated compared to those in other *Nepenthes* species. Almost no entire bodies were present, but only pieces of exoskeleton. Identification was often only possible by comparing the remains of head capsules and body parts with insects collected alive on the nectariferous parts of the pitcher plant. The dipterans were not sufficiently well preserved to be identified further than at the order level and this was often true for the coleopterans, as well. Only the ants were distinguished to morphospecies and identified to genus, when possible, using the identification key of Hölldobler & Wilson (1990).

Results

Absence of dissuasive patrols of Camponotus schmitzi towards nectar visitors

We observed on average 4.8 ± 3.8 (mean ± SD given hereafter, on 67 pitchers), nectar visitors (4.5 ± 3.9 ants and 0.3 ± 1.0 flying insects) on the outer part of pitchers during each observation. Most of these arthropods, including ants (belonging to the genera *Crematogaster* (*Crematogaster* sp. 1: 2.4 ± 3.7), *Polyrhachis* (*Polyrhachis pruinosa*: 1 ± 1.5), *Oecophylla*, *Pheidole*, *Anoplolepis* and *Tapinoma*), midges, mosquitoes and lepidopterans were found feeding on nectar of the lower face of the lid (Figure 1b). By contrast, no *C. schmitzi* workers were found walking on the pitcher body, although these ants were present on each of the pitchers observed, hidden under the peristome all around its edges (13.2 ± 6.3 workers, ants counted on a subset of 10 out of the 67 pitchers, Figure 1d). The digestive liquid had a mean pH of 4.9 which was rather constant despite pitcher age differences (SE = 0.05, N = 67 pitchers).

Effect of Camponotus schmitzi *on ant retention*

Camponotus schmitzi-occupied pitchers retained about three times more *Polyrhachis pruinosa* ants (mean = 26%, SE = 6.8%, 50 ants) than did the same pitchers when deprived of their symbiotic ant (mean = 8%, SE = 3.7%, 50 ants), as shown by the logistic regression (fixed effect of the *C. schmitzi* treatment: $F_{1,98}$ = 4.7, P = 0.03, no random effect of pitcher: variance estimate = 0.08 *vs*. residual = 1.08).

Indeed, when *Polyrhachis pruinosa* ants attempted to escape from the digestive liquid, they were in almost all cases (47 ants out of 50) attacked by *C. schmitzi*, which ambushed them from under the peristome. We regularly observed one or several *C. schmitzi* ants biting the legs of *Polyrhachis* ants. The small *C. schmitzi* ants mainly attack the intruders from the peristome, the curved and sharp teeth of which form a protective shield, but they sometimes also leave their refuge to attack the intruder from underlying parts of the pitcher (Figure 1e, on-line video clip, http:// umramap.cirad.fr/amap3/cm/index.php?page=films). As a consequence of these attacks, the *Polyrhachis* ants slipped more often in *C. schmitzi*–occupied than in *C. schmitzi*-deprived pitchers, in which they only had to cope with the slipperiness of the pitcher walls and with the previous lubrication of their tarsal pads by the digestive fluid (Poisson regression on number of falls: $F_{1,98}$ = 15.8, $P < 0.0001$; no random effect of pitcher: variance estimate = 0.001 *vs*. residual = 3.08, Figure 2a). The ants that did not escape from the digestive liquid usually died from exhaustion and drowning following numerous unsuccessful attempts to escape from the pitcher and/or numerous bites from *C. schmitzi*. Furthermore, when the *Polyrhachis* ants succeeded in escaping the pitchers within the 5-min observation time, the time required for them to escape was significantly longer in the presence of *C. schmitzi* ants (mean ± SD = 127 ± 75 s, range = 12-282 s, N = 37) than when these ants were absent (83 ± 64 s, range = 3-250 s, N = 46). Time required to escape also significantly varied among pitchers (mixed-model ANOVA on time to escape: fixed effect of *C. schmitzi* treatment: $F_{1,77}$ = 9.01, P = 0.004; random effect of pitcher: $F_{4,77}$ = 3.52, P = 0.01). *Polyrhachis pruinosa* used in the bioassays, one of the two most common prey species, was also the largest prey item recorded in the analysis of prey contents and probably the hardest to catch for *C. schmitzi*. Hence our results probably underestimated the effect of *C. schmitzi* on prey retention and our tests are therefore conservative in that respect.

Feeding behaviour of Camponotus schmitzi towards dead prey in the pitchers

Camponotus schmitzi were occasionally observed to swim in the digestive liquid and pull an ant prey item, such as *Crematogaster* or *Polyrhachis*, out of the liquid. They then hauled it up to the underside of the peristome and consumed part of the dead insect, dropping into the pitcher the cuticular remains and other uneaten parts (Figure 1f, g). No flying insect was observed to be trapped in any of the 10 pitchers during the 15-d experiment.

Thus in the presence of *C. schmitzi*, on average 42.5% of the experimental *Crematogaster* ants were either entirely removed from the liquid (1 *Crematogaster* ant found underneath the peristome) or presented traces of mandibles on the remaining body parts (34 ants, SE = 13.5), and 8.7% of the

Polyrhachis ants (7 ants, SE = 4). Such dismemberments could not have resulted from plant digestion, since none of the 40 ants in the control pitchers deprived of *C. schmitzi* showed any sign of such dismemberment. Moreover, flying insects constituted only 6% of the nectar visitors and less than 1% of the prey, and we never observed any flying insects feeding on prey in the pitcher. As crawling insects were excluded from the experiment by the glue, the feeding activity on dead prey was attributed to *C. schmitzi*. Our data indicate that *C. schmitzi* fed preferentially on the small *Crematogaster* prey items (parts or entire bodies) rather than on the bigger *Polyrhachis* prey items and that there was globally more such feeding activity in some pitchers than in others (Poisson regression on number of insect items with signs of ant feeding: fixed effect of ant species: $F_{1,7}$ = 35.9, P = 0.0005, Figure 2b; random effect of pitcher: variance estimate = 1.39 *vs.* residual = 0.40).

Analysis of the Nepenthes bicalcarata *prey spectrum*

Ants were found in 100% of the traps, with up to 386 individuals per pitcher (Table 1). Among them, 13 morphospecies were identified. The most frequently represented ant species included *Camponotus schmitzi* itself (remaining heads of queens or workers were found in 100% of the pitchers), *Polyrhachis pruinosa* (small numbers of individuals found in 90% of the pitchers) and *Crematogaster* sp. 1 (in 80% of the pitchers and up to 124 individuals found in a pitcher). Large numbers of small myrmicines were more occasionally observed in the pitchers. Large numbers of termites (up to 946 per pitcher) of a *Hospitalitermes* species were found in 40% of the pitchers. Ants and termites thus constituted 98% of the prey of *N. bicalcarata*, the flies and midges 1% and the beetles 0.5% (Table 1). Hundreds of minute pellets, never found in the pitchers of other *Nepenthes* species we analysed (unpubl. data) were found in each pitcher.

Discussion

Our behavioural observations and our experiments showed that *Camponotus schmitzi* participates in the process of prey capture by its host-plant, *Nepenthes bicalcarata,* and may also contribute to the process of prey digestion. This plant-ant systematically attacks trapped visitors that attempt to escape from the pitcher and scavenges on dead prey items that it collects from the digestive liquid of its carnivorous host-plant. It consumes part of the prey within the pitcher under the peristome and returns to the digestive liquid substantial non-eaten pieces and maybe also its faeces.

Here, we report new data that may reconcile the apparently conflicting observations reported by Clarke & Kitching (1995) and Merbach *et al.* (2007) concerning the behaviour of *C. schmitzi* towards insects: the ants combine times of passivity and aggressiveness which seems to be part of a unique adaptive strategy. On one hand, the *C. schmitzi* ants were shown, at least during daytime, to be unaggressive on open, nectar-producing mature pitchers. Such an absence of territoriality is very unusual for plant-ants, which often exhibit specialised behaviours, such as systematically patrolling nectar-producing sites which dissuades intruders including herbivorous insects (Gaume *et al.* 2005, 2006; McKey *et al.* 2005), or such as pruning their host-plant neighbours to avoid competition from other ants (Federle *et al.* 2002). On the contrary, *Camponotus schmitzi* ants conceal themselves and allow numerous insects, mainly other ants, to visit and feed on pitcher nectar. Conspicuous activity of the ants would deter potential prey, reducing benefits both to the carnivorous host-plant and to its ant colony. On the other hand, our data provide further evidence that aggressiveness can be part of the behaviour of *C. schmitzi* ants but that the ants display a 'delayed' aggressiveness which only occurs once the visitors fall into the trap. Attacks by this ant prevent insects from escaping the pitcher and its experimental exclusion decreases the prey retention rate of its host-plant. Hunting by ambush from the shelter of the curled lip of the peristome, this ant has also escaped the attention of most observers. The often unnoticeable aggressiveness that they deploy from this shelter might explain why in other studies, insects attempting to escape from the pitcher had so much difficulty in crossing the peristome (Bohn & Federle 2004). The behaviour of *C. schmitzi* is quite similar to the ambushing behaviour of a tiny Amazonian myrmicine *Allomerus decemarticulatus*, which constructs holed platforms on its host-plant, *Hirtella physophora*, to catch and kill large insects (Dejean *et al.* 2005). Complementary experiments with other prey species should provide further information on the effect of *C. schmitzi* on its host plant efficiency at capturing diverse prey.

Although the reciprocal effects on fitness of the two partners will not be easy to demonstrate experimentally, mainly because long-term exclusion of the ants would also imply an exclusion of potential crawling prey, we provide here several results which support the hypothesis of a nutritional mutualism between *N. bicalcarata* and its ant. Our results clarify the benefits of the interaction for the ants. In addition to nesting structures and sugar-rich nectar offered by the plant, our results show that the diet of *C. schmitzi* includes a regular protein meal obtained from plant-trapped prey. Indeed, the ants not only feed on large prey items, as already observed by Clarke & Kitching (1995) but also (and probably more often) on smaller ones, as supported by our insect bioassays. Although the retention experiments focused on one type of prey - the biggest and probably the most difficult to trap -, our results also suggest that the plant benefits from a food surplus provided by the symbiotic ants through their hunting behaviour. The ants only eat a small part of the prey and drop the non-metabolised parts into the pitchers. Furthermore, their activity of prey-breakdown and their metabolic activity should facilitate nutrient assimilation by the plant as does the living infauna of the digestive liquid (Beaver 1983).

It is probably not a coincidence that ants and termites were found to be abundant and numerically dominant among the prey items of *N. bicalcarata*. Indeed, they are typically the kind of prey that can be easily targeted by *C. schmitzi*, which is probably much less effective against flying insects, which do not need to climb on the inner wall or on the slippery peristome to escape from the pitcher. Termites of the genus *Hospitalitermes* (Nasutitermitinae) are nocturnally active processional termites (Jones & Gathorne-Hardy 1995). This is probably why we did not record them as pitcher visitors. They forage in large numbers and climb at night on the surrounding vegetation in search of food. Since they are virtually blind, they should be attracted by the odour cues of the pitchers, probably as in *N. albomarginata* (Merbach *et al.* 2002). However, because of their nomadic way of life, they are likely to be less reliable prey than ants, although termites have been recorded in the pitchers of *N. bicalcarata* (Cresswell 2000). Since ants, the most consistently reliable prey of *N. bicalcarata*, have been estimated to provide 70% of the nitrogen used in several *Nepenthes* species (Moran *et al.* 2001, Schultze *et al.* 1997), the additional proteins provided to the plant by the hunting behaviour of *C. schmitzi* should represent a fitness advantage for the host-plant in the nutrient-poor environments in which it occurs. In addition, the plant could probably benefit from direct myrmecotrophy since dead bodies of *C. schmitzi* were systematically found in the pitcher contents (Table 1). Because these ants rarely leave their pitcher refuge, their faeces are likely dropped into the digestive liquid and could constitute another source of nutrients assimilable by the plant. However, the ant-origin of the observed pellets in the digestive fluid remains to be demonstrated by further experiments and its participation to the plant nutrition needs to be demonstrated by isotopic analyses. Added to the anti-herbivore (Merbach *et al.* 2007) and anti-putrefaction (Clarke & Kitching 1995) benefits mediated by the ant, this nutritional benefit provided by the ant supports the hypothesis of a multi-faceted mutualism between this symbiotic ant and its carnivorous host-plant.

The association with a hunting plant-ant also constitutes a novel specific trapping strategy in the genus *Nepenthes*, which already displays a large spectrum of insect-trapping devices. The peristome was demonstrated to be very wettable in this species and to be the major surface responsible for insect fall due to aquaplaning following rain or nectar secretion (Bohn & Federle 2004). We never saw any nectar spreading over the peristome of *N. bicalcarata* during our study time, contrary to other *Nepenthes* species such as *N. rafflesiana*, but we showed that the concealed ants play a crucial role in preventing the ascent of the peristome of *N. bicalcarata* by trapped insects. Moreover, the association with a hunting ant makes sense in relation to the absence in *N. bicalcarata* of costly and efficient trapping features such as a slippery waxy layer made of long-chain aldehydes (Gaume & Di Giusto 2009), or a viscoelastic fluid made of giant polymers (Gaume & Forterre 2007).

We confirm the results reported by Clarke & Kitching (1993) on a smaller sample of pitchers that the digestive fluid of *N. bicalcarata* is less acidic than the fluid of several of its congeners, such as *N. rafflesiana* (Bauer *et al.* 2009), *N. alata* (An *et al.* 2002) or *N. gracilis* (Clarke 1997), all of which have been shown to have a pH close to 2.5, optimal for the activity of nepenthesin proteases (Athauda *et al.* 2004). This elevated pH might have an adaptive significance and protect the plant-ant symbiont

(Moran *et al.* 2010) but it also suggests that *N. bicalcarata* might not have the same pool of enzymes as the other *Nepenthes*. As *N. bicalcarata* has one of the richest and most diverse aquatic infauna in the genus (Clarke & Kitching 1993, Cresswell 2000), it might be heavily dependent on its infauna, and on *C. schmitzi*, to digest its prey. The additional work of pre-digestion by *C. schmitzi* could also compensate for a potentially weak digestive efficiency of the plant itself, due to the low acidity of its digestive fluid. Furthermore, the very long life span of *N. bicalcarata* pitchers and the plants' large size (Clarke 1997) are undoubtedly advantageous for their ant inhabitants, as they provide reliable nest-site and food resources for these permanent residents. It is tempting to hypothesize that these traits have evolved in the context of coevolution between the two partners. Long-term experiments need to be carried out to test these hypotheses and to assess whether the ant-plant mutualism is the outcome of reciprocal adaptation of the two partners.

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Tables

Prey contents of *N. bicalcarata* pitchers	**Mean ± SD number of individuals per pitcher**	**Prevalence (% of pitchers where the taxon was present)**
Hymenoptera		
Ants (Formicidae)		
Formicinae		
Camponotus schmitzi (queen + worker)	6.1 ± 4.7	100
Camponotus sp.	5.2 ± 7.9	50
Polyrhachis pruinosa	1.5 ± 1.1	90
Polyrhachis sp. 1	1 ± 1.1	60
Polyrhachis sp. 2	0.3 ± 0.5	30
Anoplolepis gracilipes	0.1 ± 0.1	10
Myrmicinae		
Crematogaster sp. 1	27.2 ± 39.2	80
Crematogaster sp. 2	10.7 ± 14.8	60
Pheidole sp.	14.8 ± 41.2	20
Myrmicinae sp. 1	1.6 ± 5.1	10
Dolichoderinae		
Tapinoma sp. 1	25.7 ± 81.3	10
Tapinoma sp. 2	15.1 ± 35.5	20
Dolichoderinae sp. 2	8.6 ± 16.4	30
Unidentified ants	1.4 ± 2.3	40
Total ants	119 ± 123.1	100
Apidae sp.	0.2 ± 0.4	20
Vespidae sp.	1.0 ± 2.2	30
Total Hymenoptera	120 ± 124	100
Total Lepidoptera (larva)	0.1 ± 0.3	10
Total Diptera	2.9 ± 3.0	80
Isoptera		
Termidae (Nasutitermitinae)		
Hospitalitermes sp. soldier caste 1	118 ± 207	40
Hospitalitermes sp. soldier caste 2	53.8 ± 169	20
Hospitalitermes sp. worker caste	141 ± 219	40
Total Isoptera	312 ± 412	40
Total Coleoptera	2.2 ± 2.0	80
Araneae (Salticidae sp.)	0.2 ± 0.4	20
Total prey	438 ± 374	100

Table 1: Prey composition of 10 pitchers of *Nepenthes bicalcarata* from a mixed peat swamp and heath forest of Brunei Darussalam (Northern Borneo).

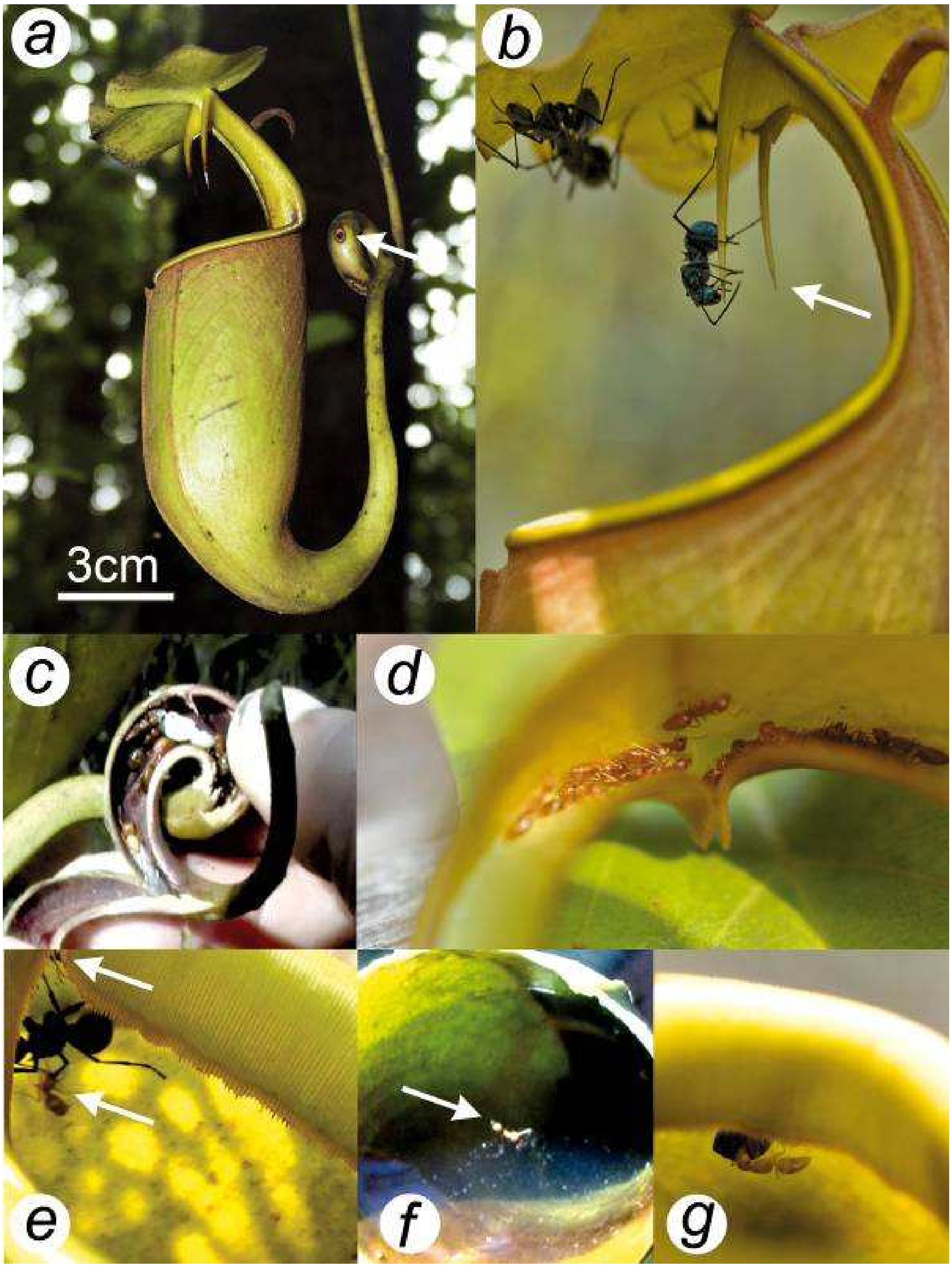

Figure 1: Overview of the ant-plant interaction. Upper pitcher of *Nepenthes bicalcarata*. The arrow indicates the domatium within the coiled tendril which harbours *Camponotus schmitzi* ants (a). *Polyrhachis pruinosa* workers feeding on the extrafloral nectar produced by the pitcher on the inner side of the lid and by the two thorns (arrow) surrounding the pitcher (b). Sectioned domatium showing workers, alates and brood of *Camponotus schmitzi* (c). *Camponotus schmitzi* workers in ambush position under the peristome (d). A fallen *Polyrhachis pruinosa* ant being attacked (arrows) by two *Camponotus schmitzi* (e). *Camponotus schmitzi* (arrow) can safely swim in the digestive liquid from which it removes prey items (f) that are then consumed under the peristome (g).

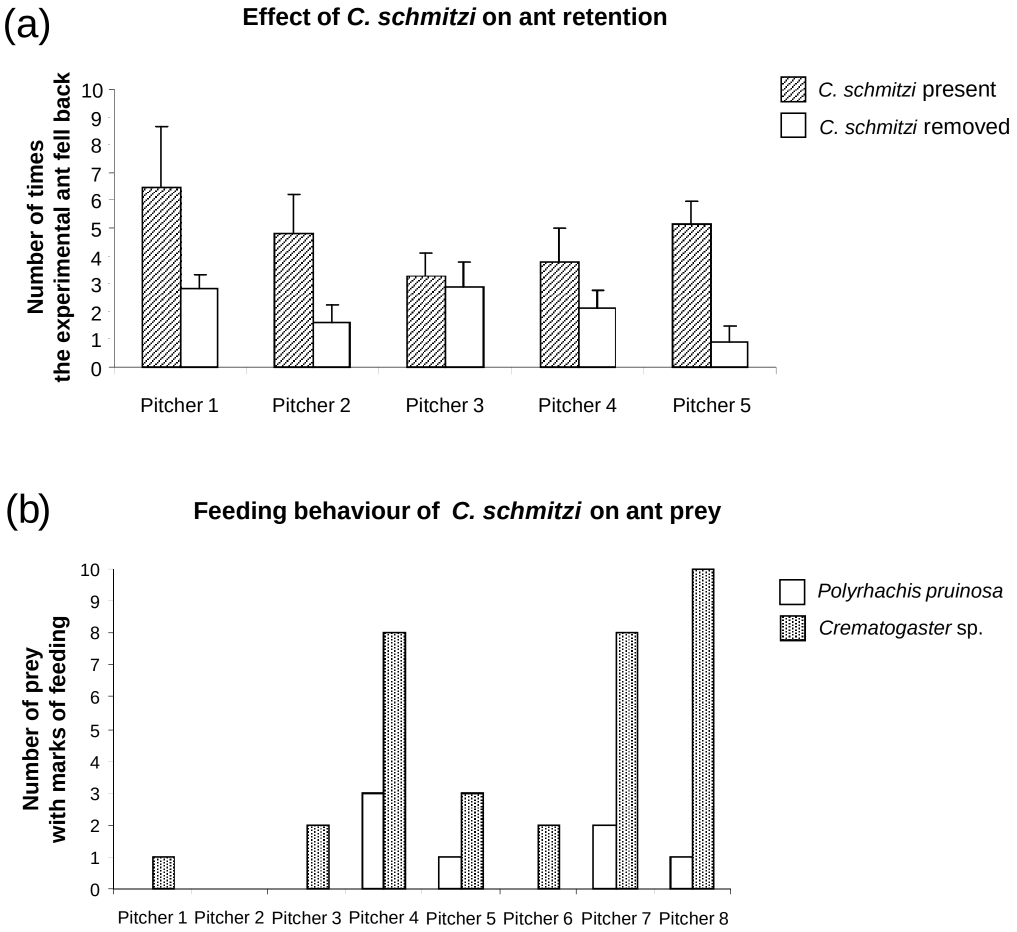

Figure 2: Experimental results showing the effect of the ant symbiont on both prey retention and breakdown in *Nepenthes bicalcarata*. Effect of *Camponotus schmitzi* on ant retention (a). The x-axis refers to the different experimental pitchers. The y-axis refers to the number of times the experimental ant fell back into the digestive liquid, attempting to escape from the pitcher, during a 5-min session. The error bars refer to 1 SE. Evidence of feeding behaviour of *Camponotus schmitzi* on dead ants of two different sizes (b). The x-axis refers to different experimental pitchers. The y-axis refers to the number of prey (out of 10) which bore marks of ant feeding and breakdown after the 15-d experiment. No prey breakdown was observed in the control pitchers (not shown) where *C. schmitzi* was excluded.

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Chapitre I. 3

UN MÉCANISME DÉVELOPPEMENTAL D'ÉVITEMENT DU CONFLIT POLLINISATEUR-PROIE

Toutes les plantes carnivores sont entomogames et l'utilisation des insectes comme proies et pollinisateurs crée un conflit évolutif. Le genre *Nepenthes* est le seul genre de plantes carnivores à être dioïque ce qui interdit toute possibilité de reproduction sexuée autogame. Quelques études se sont intéressées à ce conflit et à sa résolution chez d'autres genres, et mis en évidence des recouvrements des guildes de proie et de pollinisation. La plupart des mécanismes d'évitement proposés, vraisemblables mais rarement étudiés, reposent sur un décalage temporel ou physique de l'activité des organes carnivores et sexuels. Le **Manuscrit I.3** teste chez *Nepenthes rafflesiana* var. *typica* si un mécanisme développemental permet de minimiser ce conflit. Nous avons comparé la phénologie des organes carnivores chez des individus qui fleurissaient et d'autres qui ne fleurissaient pas. Le nombre d'urnes actives diminue-t-il durant la floraison ? Sont-elles plus éloignées de l'apex de la plante, où la floraison terminale se produit, pendant la floraison ? La floraison implique-t-elle une diminution de l'activité du compartiment carnivore ?

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STOP-AND-GO DEVELOPMENTAL STRATEGY MINIMIZES THE POLLINATOR-PREY CONFLICT IN THE CARNIVOROUS PLANT *NEPENTHES RAFFLESIANA*

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Abstract

Carnivorous plants face a dilemma as regards to the use of insects: they capture and digest them to meet their nutrient needs but also rely on them for pollination service. Though overlooked, this nutrition-pollination conflict is suspected to have been evolutionary prevented in many carnivorous genera by the set-up of spatial and/or temporal separations of the carnivorous and reproductive organs. In this study we explore whether a spatial and/or a temporal separation of the carnivorous and pollination function occurs in a climbing pitcher-plant of the carnivorous genus *Nepenthes*. During a field study in the heath forests of Brunei (Borneo), we compared in *Nepenthes rafflesiana* the developmental dynamics of the carnivorous traps between blooming and not-blooming individuals. We showed that the development of the buds towards future traps is paused during the flowering time. Functional traps are also fewer during blooming and located far away from the flowers. When the blossom period ends, the development of the traps resumes synchronously which may confer a nutritional boost for the synchronous sympodial branching and fructification needs. This stop-and-go developmental pattern thus minimizes the prey-pollinator conflict in the *Nepenthes rafflesiana* carnivorous plant without decreasing the number of traps produced.

Keywords: Pollination strategy, evolutionary conflict, pitcher-plant.

Introduction

Carnivorous plants grow in low-nutrient soils and complete their nutrition with nutrients obtained from the digestion of insects they attract and capture (Juniper *et al.*, 1989). Since most of the carnivorous plants are also insect-pollinated (Ellison & Gotelli, 2001), it may lead to a plant's dilemma for the use of insects as both pollen vectors and nutrient providers (Juniper *et al.* 1989; Givnish et al. 1989; Ellison & Gotelli, 2001) so far called pollinator-prey conflict (Zamora, 1999). However, as little is known on the pollination biology of most of the carnivorous plants, actual evidence of such a conflicting situation is scarce and the way it has been solved or at least minimized has been largely overlooked. Among the few studies on the topic, Zamora reported some overlapping between pollinator and prey guilds of the butterwort *Pinguicula vallisneriifolia,* which was therefore shown to suffer from pollen limitation in peculiar environments (Zamora, 1999). Murza *et al.* later showed that the sundew *Drosera anglica* produces long-peduncle inflorescences which exploits an insect guild different from that captured in its sticky carnivorous-leaves (Murza *et al.*, 2006). The evolution of such long-peduncle flowers in *Drosera* but also in the genera *Pinguicula* and *Cephalotus* can be viewed as a mechanism of spatial separation between flowers and traps to minimize the pollinator-prey conflict (Juniper et al. 1989, Murza et al. 2006). However, an alternative hypothesis to such a 'pollinator protection hypothesis' proposed that this long-peduncle and thus conspicuous flowers could have been selected as a 'pollinator attraction mechanism' (Anderson & Midgley, 2001, Anderson, 2010). In addition to such possible spatial mechanisms, temporal separation between reproductive and carnivorous organs has also been proposed to minimize the pollinator-prey conflict. For example, most of the *Sarracenia* species flowers before new trap leaves appear (Juniper et al. 1989). However, no avoidance mechanism of the pollinator-prey conflict has been proposed nor investigated in pitcher plants of the *Nepenthes* genus yet (Juniper *et al.*, 1989).

The one hundred species of the *Nepenthes* genus are all carnivorous and climbing plants, mainly distributed in South-Eastern Asia, with Borneo and Sumatra as hotspots of species diversity (Juniper *et al.*, 1989, Clarke, 1997, Clarke, 2001, McPherson, 2009). Unlike in other carnivorous genera, photosynthetic and carnivorous organs, i.e. leaf blades and pitchers respectively, are spatially separated by a tendril. Their respective developments are also uncoupled: pitchers buds start to differentiate only once the leaf blades bearing them have ended their own development. Then during a few weeks, they develop a closed cavity in which a digestive liquid is secreted (Clarke, 1997). They finally open and become fully functional a days after the opening of the lid (Bauer *et al.*, 2009). A well-known combination of adaptations that include attraction devices such as flower-mimicking UV guides (Moran *et al.*, 1999), floral scents (Di Giusto *et al.*, 2008, Di Giusto *et al.*, 2010) and nectar (Moran, 1996) as well as capture devices such as slippery surfaces (Juniper & Burras, 1962, Gaume *et al.*, 2002, Bohn & Federle, 2004, Gaume *et al.*, 2004, Bauer *et al.*, 2008), viscoelastic fluids (Gaume

& Forterre, 2007, Di Giusto *et al.*, 2008) or even hunter ants (Bonhomme *et al.*, in press) are involved in the trapping system of these carnivorous pitcher plants.

By contrast, the pollination biology of the *Nepenthes* genus is poorly known (Juniper *et al.*, 1989). Sexuality is acquired when the vine enters into the climbing phase and then produces inflorescences at the apex of the sympodial axis, whose structure (raceme or panicle) and length vary between species (Clarke, 1997, McPherson, 2009). Being dioecious, all *Nepenthes* species are obligate outbreeders (Cheek & Jebb, 2001). The tetrad-form of pollen suggests entomophilous pollination (Kaul, 1982, Adam, 1998). Nectar seeping from the tepals and sweet scents contribute to attract insect pollinators (Juniper *et al.*, 1989, Kato, 1993). The pollination habits of only a couple of species have been studied (Kato 1993; Adam 1998). While the fetid odour of the flowers of some peculiar montane species suggest myophily (Kaul 1982), the putative pollinators of most other species are flies but also moths, butterflies and wasps (Kaul, 1982, Adam, 1998, Frazier, 2001) and maybe also coleopterans in the case of *N. rafflesiana* (Moran 1996). A few individuals of the same species of moths and flies (Di Giusto et al. 2008, 2010) were found in the insect spectra of both flower visitors and pitcher victims of *Nepenthes rafflesiana*, which supports the hypothesis of the existence of a pollinator-prey conflict in this species.

This study investigates whether some developmental mechanisms in the *Nepenthes* genus minimize the pollinator-prey conflict: does the number of active pitchers decreases and/or does the distance between sexual and carnivorous organs increases when flowering? We compared the activity and development of carnivorous organs, i. e. pitchers, for flowering and non-flowering individuals and discuss whether a phenological barrier has been selected to minimize the pollinator-prey conflict.

Materials and Methods

The study was carried out in the coastal sandy soils of Brunei Darussalam, northern Borneo (4°34’ N, 114°25’ E), in July-August 2009 on *Nepenthes rafflesiana* var. *typica* Jack. This species is particularly interesting to investigate the prey-pollinator conflict since it has already been shown to capture a broad diversity of flying insects (and thus putative pollinators) (Di Giusto *et al.*, 2008, Gaume *et al.*, 2007) and to produce pitchers that secrete nectar and scents that mimic floral parts (Bauer *et al.*, 2009, Di Giusto *et al.*, 2010).

The development of carnivorous traps was compared between flowering and non-flowering individuals in a 300 × 100 m rectangular area. Thirty two flowering and 33 non-flowering (including 15 and 16 males respectively) sexually mature individuals (i.e. that have already flowered) were identified. The non-flowering individuals did not present any sign of weak physiological condition such as yellowing leaf blades, recently damaged axes, marks of herbivory, etc. The developmental stages of pitchers developed at the six most apical nodes were recorded for the 65 individuals. This 6-node limit has been chosen because we never found any functional or developing pitcher below the sixth node. We distinguished four developmental stages for the pitchers: developing; open (the only stage that can trap insects); no longer functional and aborted (which had stopped its development in the bud stage). We then measured three partly correlated variables to characterize the distance separating the apical node from the first active pitcher: the number of nodes, the length along the axis, the absolute length to the pitcher aperture. Measuring distances from the apical node, where the inflorescence occurs, allowed us to obtain homologous distance measurements between flowering and non-flowering individuals. One month after flowering, when the fruits were maturing, we estimated for each functional pitcher and for each individual the duration of activity of the functional pitchers. As we conducted on the same period a survey of pitcher activity throughout their lifespan, we established a frame of reference (Table 2) and relied on it to estimate the number of weeks since the opening of the lid. To compare the synchronicity of pitcher opening within an individual, we calculated for each individual bearing at this time at least two functional pitchers, the variance of the pitchers' age estimates.

Statistical analyses were carried out using generalized linear models (GLM) performed with the software package R (R Development Core Team, 2009). The count data (the number of pitchers at each developmental stage described above and the number of nodes) and continuous data (the distances) were respectively analyzed using Poisson and Normal error distributions. We tested the effect of plant phenology and plant sex on these variables using factor 'flowering' and 'sex'. The variances in pitchers' ages were compared using Welch's *t*-tests that do not make any assumption of variance homogeneity.

Results

In *Nepenthes rafflesiana* var. *typica*, the number of functional pitchers is significantly ($P < 0.012$) lower during flowering (Flowering plants: 1.31 ± 0.56 pitchers, mean and SD given throughout the text, N = 32; Non-flowering plants: 2.36 ± 1.06 pitchers, N = 33). No difference between sexes ($P = 0.670$) in the number of functional pitchers was observed either for flowering or for non-flowering individuals (Table 1, Figure 2). The number of developing pitchers were observed to be more numerous within the group of flowering individuals, most of them being at the end of the "inflating bud" stage (Flowering: 1.97 ± 1.18 developing pitchers, n = 32 individuals; Non-flowering: 3.50 ± 1.32 developing pitchers, n = 33 individuals – $P < 0.0001$). Some pitchers were found aborted and others no longer functional, but without any difference according to the 'flowering status' of the plants ($P = 0.10$). Thus the only difference observed between flowering and non-flowering groups was the developing pitchers / functional pitchers ratio, which appears to be four times higher for flowering individuals. The total numbers of aborted and non-aborted pitchers were otherwise similar between the flowering and non-flowering groups of plants.

Whatever the type of measure of the apex-pitcher distance used, the first active pitcher is found located further from the apex when the plant is flowering. An apex-pitcher distance of 4.6 ± 1.4 internodes was observed for the flowering individuals and 2.37 ± 1.06 internodes for the non-flowering individuals ($P < 0.001$ – Table 1). For the distance along the axis between apex and pitcher we respectively measured 232.9 ± 134.0 mm and 127.0 ± 94.9 mm ($P < 0.001$) and 421.3 ± 196.9 mm and 273.1 ± 117.4 mm ($P < 0.001$ – Table 1, Figure 2) for the absolute distance. For each of these three variables, no significant difference was observed between sexes either within flowering or non-flowering individuals (Table 1).

Our estimations of activity stage of the pitchers indicate, through a lower variance observed for individuals that have flowered, that they became functional more synchronously than non-flowering individuals (Variance of activity stage for fruiting individuals: 0.63 ± 0.72 weeks^2, n = 23 individuals; non-flowering individuals: 1.75 ± 1.23 days^2, n = 31 individuals – Welch's *t*-test = 4.029, $P > 0.001$).

Discussion

We report here a phenology of pitcher production that minimizes the pollinator-prey conflict in *Nepenthes rafflesiana*: during the flowering period, fewer pitchers are active, they are located further away from the apex and thus from the inflorescence. The risk to be trapped inside the pitcher for the pollinators is thus reduced. Such a spatial separation of pitchers and flowers actually results from a temporal separation of pitchers and flowers in their timings of production: the pitcher follows a stop-and-go development pattern: its development is paused during blooming and resumed when the blooming ends. The development of new carnivorous traps is paused rather than aborted, and as the developing pitchers then all synchronously open when the flowering ends, the total number of traps produced during the reproductive period is thus not reduced compared to non-flowering plants during the same period of time. Such a developmental pattern could be viewed as a plant phenological mechanism that has evolved to alleviate the pollinator-prey conflict.

One may object that during flowering more resources need to be allocated to the reproductive organs than to the carnivorous ones, and thus that the differences observed strictly result from the outcomes of a cost/benefit investment strategy of the plant at a given period of its life. Direct monitoring is needed to clarify this point but it seems that the pitchers are paused at a relatively late stage, probably when most of the production costs have already been paid and that the pitcher then just elongate. This might especially be true for female plants which produce fruits and which thus need more resource than males but we did not find any significant differences in the pitcher developmental strategies of male and female plants. This objection would neither explain why pitchers were paused at the same developmental stage nor why they synchronously resume their development. This also suggests that the inflorescence plays a central role in the regulation of this mechanism and it would be interesting to test whether flower hormones (directly or not) inhibit the pitcher development.

This original stop-and-go developmental pattern allows the plant to minimize the pollinator-prey conflict without affecting the global intake of arthropod-derived nutrients. Indeed, the activity of carnivorous organs is delayed rather than decreased, the delay being later compensated by a prompt and synchronous resumption at a relevant period. Moran has shown on a small sample size that covering pitchers and thereby preventing them to catch insects before the flowering process did not affect the amount of seeds produced, probably because the plants can rely on some reserves (Moran, 1991). This point would have to be confirmed but the synchronous opening of the previously paused–pitchers probably provides an advantageous boost of key-nutrients for the fructification process and for the sympodial branching. An exhaustive field study of the pollination and reproductive biology of the genus *Nepenthes* needs to be carried out to get an integrative approach of the evolutionary ecology of this carnivorous plant and to fully assert how it minimizes its pollinator-prey conflict.

Tables

Variability in the number of functional pitcher and their distance to the apex

Response variable: number of functional pitchers	df	χ^2	P(> χ^2)
Phenology	1	6.548	0.012
Sex	1	0.184	0.668
Number of nodes between the apex and the first functional pitcher	**df**	χ^2	**P(> χ^2)**
Phenology	1	12.659	<0.001
Sex	1	0.186	0.666
Distance along the axis from the apex to the first functional pitcher	**df**	**F**	**P(> F)**
Phenology	1	13.855	<0.001
Sex	1	0.923	0.340
Absolute distance from the apex to the first functional pitcher	**df**	**F**	**P(> F)**
Phenology	1	13.492	<0.001
Sex	1	0.024	0.878

Table 1 : Number of functional pitchers and their distance to the apex of the plant, where the inflorescence occurs, compared using analysis if variance for flowering and non-flowering plants.

Week after opening	Pitcher body	Lid	Peristome	Digestive liquid
1st	green or pale yellow, still growing	very flexible, becomes horizontal moving away from the peristome	developing outwards on both sides of the pitcher rim, soft, not very coloured, when present coloured bands do not strongly contrast	very limpid, very viscoelastic, acidity increasing
2nd	yellow or green, definitive size reached	horizontal, begin to secret nectar and odours	fully developed, strong odour production, almost always covered by nectar	limpid, very viscoelastic, no particular smell, maximal acidity
3rd	duller yellow-green, some red or brown spots appear	horizontal, secrets nectar and odours	strong smell, strong nectar production	light pink or brown coloration of the liquid, less viscoelastic, light putrefaction smell
4th	red or dark spots always present, outer walls of the pitcher are often covered by a mould layer	sometimes eaten, lower nectar production, sometimes hunched	lower levels of nectar and odor production	rose or brown coloration, viscoelasticity of the liquid very low often undetectable with fingers, acidity starts to decrease
5th	a lot of dark spots are visible, covered by a mould layer	often predated, dark margin, becomes darker often hunched	sometimes spotted, dried or drying	strong putrefaction smell, very turbid liquid made of insects residuals, non-viscoelastic digestive liquid

Table 2: Frame of reference for the dating of pitcher age after its opening.

Figures

Figure 1: This photograph has been made one month after the end of blooming (a growing infructescence is shown within the ellipse) as estimated by the development of the infrutescence. Arrows indicate three pitchers with similar functional stage (in their third week after opening). This indicates that they became functional almost at the same moment, just after the flowering time. This synchronicity contrasts with the 'continuous' and more gradual development they exhibit when the plant is not flowering.

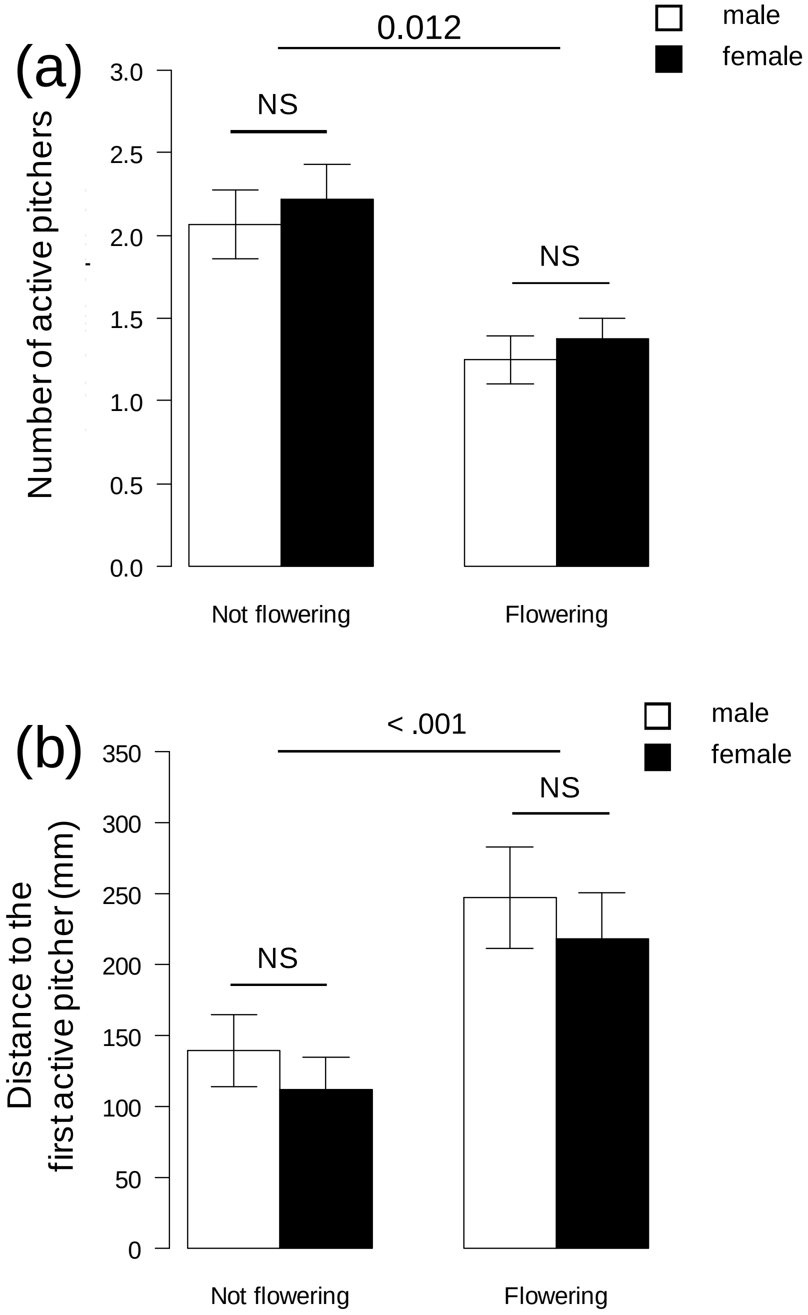

Figure 2: During the flowering period less pitchers are active and they are located further from the apex than in non-flowering period. (a) The number of active pitchers in *Nepenthes rafflesiana* is lower when the plant is flowering (P < 0.012) and there is no difference between the sexes, whether the plant is flowering or not. (b)The absolute distance from the apical node (where the inflorescence occurs) to the node bearing the first active pitcher is higher when *Nepenthes rafflesiana* flowers (P < 0.0001), and there is no difference between sexes.

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Chapitre II

HISTOIRE ÉVOLUTIVE DU GENRE ET DES MÉCANISMES DE PIÉGEAGE

Chapitre II.1

PHYLOGÉNIE MOLÉCULAIRE DU GENRE *NEPENTHES*

Disposer d'une phylogénie résolue permet de comprendre les relations évolutives entre espèces et les dynamiques de diversification des lignées. L'objectif de cette thèse de retracer l'histoire évolutive de caractères d'intérêts (par ex. perte de la zone cireuse, viscoélasticité du liquide) et de comprendre la diversification du genre en lien avec les écologies des espèces était subordonné à l'amélioration des phylogénies actuellement disponibles. Le **Manuscrit II.1** présente une phylogénie plus complète du genre basée sur des marqueurs AFLP, quatre gènes chloroplastiques et un gène nucléaire, discute sommairement l'histoire biogéographique du genre et les regroupements taxonomiques précédemment établis.

Bonhomme, V., Gaume, L., Ardisson, M., Clamens, A.-L. & Jousselin, E. (in prep.) An updated molecular phylogeny of the genus *Nepenthes* based on ITS, cpDNA and AFLP.

AN UPDATED MOLECULAR PHYLOGENY OF THE GENUS *NEPENTHES* BASED ON ITS, CPDNA AND AFLP

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Abstract

The genus *Nepenthes* (Nepenthaceae) encompasses at least 120 species, all carnivorous and mainly distributed in SE Asia. In spite of a growing interest in their ecology and particularly the trapping mechanisms of their pitcher-modified leaves, we still lack a robust phylogenetic reconstruction to test evolutionary hypotheses and infer biogeographic scenarios for this genus. The phylogeny of the genus has been investigated for ten years but low genetic variation for the chloroplastic and nuclear DNA markers sequenced so far has lead to poorly resolved phylogeny. We here report an updated phylogeny based on nuclear ITS, four new chloroplastic DNA markers and AFLP data. The latter have been shown to be useful for phylogenetic reconstructions at the genus level for plants but our analyses yield very ambiguous results for *Nepenthes*. Yet not fully resolved, the phylogenies obtained, based on cpDNA and ITS data indicate some geographical structuration of the genus and clarify relationships for some species from N/W Sumatra and Borneo, recognized as hotspots of *Nepenthes* diversity. Previous attempts of infrageneric taxonomic subdivisions are partly invalidated by our results[2]

Keywords : Nepenthaceae, pitcher plants, SE Asia

Introduction

The genus *Nepenthes* belongs to the monotypic family Nepenthaceae (Caryophyllales) and encompasses at least 120 species mainly distributed in SE Asia, with Borneo and Sumatra recognized as hotspots of biodiversity (Clarke, 1997, Clarke, 2001, McPherson, 2009). All the species of the genus are carnivorous and derive key-nutrients from their pitcher-modified leaves that trap and digest arthropods, circumventing the nutrient shortage of the environments they colonize. In spite of the growing interest in their ecology, the evolution of their trapping mechanisms and physiology, the systematics of the group and species evolutionary relationships within the genus are not resolved. Beyond its taxonomic interest, a robust phylogenetic reconstruction would allow to reconstruct the biogeographic history of the genus.

Solving phylogenetic relationships at infrageneric level is generally difficult for plants (Shaw *et al.*, 2005). This is also the case in the genus *Nepenthes*, which phylogeny has been investigated with molecular data since 2001. The first molecular phylogeny relied on chloroplastic *trnK* intron (Meimberg *et al.*, 2001) which brought a poorly resolved phylogeny yet sufficient to partly discard previously proposed infrageneric classification (Danser, 1928) and to show that the western species (*N. khasiana* from the Indian Khasi, *N. distillatoria* the type species of the genus endemic to Sri Lanka, *N. pervillei* endemic to the Seychelles and the two malagasian *N. masoalensis* and *N. madagascariensis*) group together in a basal clade. Meimberg et *al.* later analyzed a translocated copy of this *trnK* intron which translocation event seem older than the radiation of modern *Nepenthes* but which was neither enough informative for the phylogenetic reconstruction of the genus (Meimberg *et al.*, 2006). Meimberg and Heubl then analysed a nuclear marker, the peptide transferase 1 (*PTR1*) which confirmed patterns obtained with independant cpDNA but resolved only a few clades (Meimberg & Heubl, 2006). The results of these studies are summarized on Fig. 1-*trnK* and Fig. 1-*PTR1*.

In this study we used additional sequence data to further investigate phylogenetic relationship within the genus. Four other chloroplastic markers (*rps16*, *trnD*, *trnL*, *ycf6*) were used as well as the nuclear internal transcribed spacer (ITS). We also investigated the potential of amplified fragment length polymorphism (AFLP) analyses for the reconstruction of phylogenetic relationships within the genus. AFLP has been increasingly used for phylogenetic reconstruction of closely related organisms for which sequence data contains low variability and it has been proved informative in several plant genera (Despres *et al.*, 2003, Koopman, 2005). The phylogenetic clusters identified by our reconstructions are compared with previous phylogenies and both taxonomic classifications proposed earlier and geographical groups are discussed.

Materials and Methods

Taxa sampling and DNA extraction

Leaves in silicagel were collected from 109 specimens representing 85 putative species of the genus *Nepenthes* (Table 1). All samples were collected between 2006 and 2009 in the collection, owned by Jean-Jacques Labat. Outgroups included *Drosera* sp. and *Drosophyllum lusitanicum*, the sister genera of *Nepenthes*. Total genomic DNA was extracted from a single leaf from a single individual for each sample using DNeasy Qiagen plant extraction kit (Qiagen, Germany) following the manufacturer's instructions. In order to obtain higher concentrations we also used a phenol chloroform protocol for some samples.

DNA amplification and sequencing

We amplified and sequenced DNA data from 5 different fragments. We used several intergenic regions in chloroplastic DNA: rps16, trnL, trnD and ycf6 and the nuclear ribosomal internal transcribed spacer region (ITS). All these markers have proved informative and useful in reconstructions of phylogenetic relationships at the interspecific level in several plant genera (Shaw *et al.*, 2005, Pelser *et al.*, 2007) but had not been used to investigate phylogenetic relationships within *Nepenthes* yet. List of primers used and amplification conditions are given in Table 2. PCR products were sent to sequencing services (Macrogen, South Korea). The amplification primers were also used as sequencing primers.

AFLP analyses

Genomic DNAs were checked for quality and quantity using nanodrop dosing and migration on agarose. Total DNA were digested using *Eco*RI and *Mse*I restriction enzymes at 37°C for 2 hours. *Eco*RI- and *Mse*I-linkers were then ligated to the resulting DNA fragments by incubating with a DNA ligase overnight at 4° C. Pre-selective PCR amplifications were performed using primers *Eco*RI-A and *Mse*I-C selective PCR amplification was performed using a fluorescently –labelled *Eco*RI-A primer. Eighteen primer pairs were tested (all the combinations with *Eco*RI-ACA, -ACG, - ACG and *Mse*I-CAC, - CAG, -CAT, -CTC, CTA, -CTG and 6 were retained (*Eco*RI-NNN/*Mse*I-NNN given: ACG/CTG, ACG/CAC, ACG/CAT, AGG/CAC, AGG/CAG and ACA/CAC) on the basis of the quality of chromatograms obtained. Automated scoring was performed on the resulting profiles using Genemapper V3.7 (Applied Biosystems).

Sequence / AFLP analyses and phylogenetic reconstructions

Sequences were individually checked by visual inspection. For chloroplast genes, they were verified for protein coding frame-shifts to avoid pseudogenes (Zhang & Hewitt, 1996) using Mega 3 (Kumar *et al.*, 2004). In a couple of cases, several ITS sequences from the same individual were compared to check for paralogy. Sequences were identical which suggests that paralogues were not divergent or not amplified. Sequences were aligned using Clustal X (Thompson *et al.*, 1994) with default settings. All alignments were verified and corrected “manually” by inserting/deleting gaps to minimize their numbers. Sequences and alignments are available on Genbank (Accession numbers given in Table 1).

The binary AFLP matrix was analysed using a neighbour-joining (NJ) tree based on Nei-Li distance using Paup*v4.0b10 and bootstrapped using 500 replicates. This distance only takes into consideration shared 1’s (and not shared 0’s) which is appropriate for restriction data as there are multiple ways for a band to be absent and thus shared 0’s may not be homologous.

For phylogenetic reconstructions, we used the Maximum Likelihood approach (ML) and Bayesian phylogenetic analyses on DNA sequences. The analyses were conducted separately on two sequence datasets: chloroplast DNA and ITS. Chloroplast DNA matrix was obtained by concatenating all chloroplast DNA fragments together. ML phylogenetic analyses were computed using Phylip (Swofford, 2002, Guindon & Gascuel, 2003). The model of nucleotide substitution was chosen by comparing nested models with likelihood ratio tests using Modeltest (Posada & Crandall, 1998). We also tested the addition of parameters for proportion of invariant sites (I) and for heterogeneity of substitution across sites (Γ) (Yang, 1994). The MP tree with the greatest – Ln score was used to estimate the model parameters (gamma shape, base frequencies, transition matrix). A ML heuristic search using TBR branch–swapping and a starting tree (found by neighbour-joining) was then run using the selected models and parameters. For ML analyses, node support was assessed with the bootstrap technique (500 replicates) performed on variables sites only, and on a subset of taxa (identical sequences were represented only once) to limit computational time.

Bayesian phylogenetic analyses were conducted using MrBayes 3 (Huelsenbeck & Ronquist, 2001). We used a six-parameter general time reversible model of nucleotide substitution (GTR, nst=6) and a random starting tree. The chloroplastic DNA dataset that included four DNA fragments was divided into four partitions, one for each fragment. Parameters of the model were treated as unknown variables with uniform prior probabilities and were estimated during the analysis and allowed to vary across partitions. Two replicate analyses were run, each to ensure that the runs were converging on the appropriate posterior probability distribution. We ran four chains of the Markov Chain Monte Carlo, sampling every 100 generations. The point of stationarity was then determined (when the distribution of likelihoods was seen to have reached a plateau).We had to run the analyses for 30 million generations for chloroplast DNA dataset and 2 million generation for ITS data set to reach for the runs to converge, the trees prior to stationarity were discarded and the remaining trees were used to compute 50% majority rule consensus trees. The posterior probabilities (pp) were summarized accordingly. Probabilities >90% were considered to indicate significant support.

Geographic and taxonomic groups

Geographic distribution of the species included in our reconstructions were retrieved from the review made by McPherson on the genus (McPherson, 2009). We retained the same eight geographical zones than Meimberg and Heubl (2006), namely, Borneo, Sumatra, Philippines, Malay Peninsula, Thailand/Cambodge/Vietnam/Laos, Sulawesi, New-Guinea/Australia and a “Western” group that encompasses species from Sri Lanka, Indian Khasi and Madagascar (Meimberg & Heubl, 2006). These geographic zones were simply mapped on the phylogenetic trees using the “trace history” option under the parsimony criterium in Mesquite (Maddison & Maddison, 2010). Our aim here was simply to visualize how species clustered according to geographic zones and not to infer ancestral areas from our reconstructions. As our phylogenetic trees showed many polytomies, applying analytical methods of ancestral character state reconstruction or historical biogeography would not have brought accurate results. When available, we also indicated on the trees obtained the affiliation to the infrageneric groups proposed by Danser (Danser, 1928).

Results

For ITS dataset, outgroups were far too divergent to be aligned with confidence. We therefore chose to root our trees with *Nepenthes khasiana* that has been shown in previous studies to be basal in the *Nepenthes* group (Meimberg *et al.*, 2000, Meimberg *et al.*, 2001, Meimberg & Heubl, 2006), this latter result was confirmed by our chloroplastic data analyses and AFLP data. Bayesian inference on combined dataset with compartimentation of all types of data obtained (the 4 cp DNA markers, ITS and restriction data from AFLP) were not attempted because of the lack of congruence between cpDNA, nuclear DNA and AFLP, due partly to missing data, particularly for AFLP (Table 1). We therefore chose to present the results of each dataset separately.

Chloroplast DNA

We obtained an alignment of 4089 base pairs, but not for all taxa (Table 1). Some of the markers had some long insertion-deletions that we chose to delete from the alignment (their inclusion did not change the topologies, data not shown). We obtained a Bayesian tree with low resolution (see cpDNA Tree), ML tree topology was highly similar (data not shown) . The deep nodes of the phylogeny were poorly resolved. Some species clusters though reflected the geographical proximity of species. Four well sustained clades were retrieved and are discussed below: one including all western species, one including some of the species from North/West Sumatra (clade A), another including some Bornean species (clade B) and the last one including some species from the Philippines (clade C).

Because of the lack of resolution of our phylogenetic trees and those of Meimberg et *al.* and, and also because species sampling do not exactly match each others (Meimberg *et al.*, 2001, Meimberg & Heubl, 2006), it is difficult to compare our results with previous ones. Still, some monophyletic species groups were clearly congruent with their results. For instance we confirmed that *N. reinwardtiana*, *N. macrovulgaris, N. northiana* are closely related (Clade D). On the other hand *N. hirsuta* appears closely related to this group in our results but is placed in a different species group by Meimberg and co-authors in their cp DNA phylogeny. A species group from the Philippines including the New Guinean species *N. insignis* (clade C) is retrieved by both our phylogeny and Meimberg's et *al.* (2006). However other species from the Philippines are dispatched throughout the tree. We also retrieved the Meimber's *hamata* group with our cpDNA analyses, i.e. *N. hamata*, *N. glabrata*, *N. muluensis* (Meimberg *et al.*, 2001).

Globally, in some parts of the tree, there was some clustering of species according to their geographic origin, which is congruent with previous analyses of cpDNA. For instance, the clade B encompasses some species from Borneo. However, again, Bornean species do not form a monophyletic clade and numerous Bornean species are dispatched throughout the rest of the phylogenetic tree. Clade A includes almost all Sumatran species except *N. sumatrana, N. burkei* and *N. mikei*. This clade compriseth *N. bongso* and its putative relatives or synonyms, the so-called "*bongso* group", i.e. *N. bongso* ex. *carunculata, N. bongso* ex. *sumatrana*, *N. gymnamphora* ex. *pectinata* and *N. gymnamphora* ex. *xiphioides* (Cheek & Jebb, 2001). We also confirm the Clarke's view concerning the close relationship of *N. diatas* and *N. densiflora* (Clarke, 2001). Interestingly, three species endemic to the Philippines group are clustered with species from Borneo suggesting some colonisation events between these two geographic blocks. This situation is also retrieved with *N. philippinensis*, nested in the Sumatra clade, which suggests again some repeated colonisation events between these islands. Similarly, two species endemic to Sulawesi, *N. maxima* and *N. eymae*, group with the essentially Bornean clade (clade B) which suggests a colonization of the Sulawesi island from Borneo, through the Wallace line.

ITS data

52 taxa have been successfully sequenced so far (Table 1), and we have obtained an alignment of 661 base pairs that shows more variability than the 4000 bp of cpDNA we have sequenced. We obtained a Bayesian tree (ITS tree) with relatively good resolution, the ML tree topology was very

similar (data not shown) to the Bayesian tree and was also relatively well supported. Mapping of geographical data on this tree also revealed that species sharing the same geographical distribution were often closely related and some formed monophyletic groups. We retrieved the N/W Sumatra (clade A1) geographical cluster obtained with the analysis of cpDNA, and this group (if we exclude *N. ovata and N. ephippiata*) appears monophyletic. These species are closely related (in the clade A) to clade A2 which contains species from Malay Peninsula and Cambodge/Thailand. A second relatively well-resolved clade appeared, encompassing mainly species from the Philippines (Clade C1). This clade C1 is not fully congruent with results obtained with cpDNA: *N. villosa* and *N. petiolata* seem to be related, as observed in the cp tree but group here with *N. sibuyanensis*, *N. ventricosa*, *N. merrilliana*, however this group was supported by low pp values. Complementary cpDNA data might lead to the grouping of the two clades as observed here with ITS data. In any case, this group of species from the Philippines appears closely related to species from Borneo and Sulawesi. Other species from Borneo, including again *N. maxima* from Sulawesi (clade B), cluster together at the base of the tree. Species from Sulawesi are more dispatched throughout the tree, and the Meimberg's *N. hamata* group is also retrieved from ITS dataset (clade C2). Unfortunately we did not manage to obtain ITS sequences for three western species (*N. distillatoria*, *N. madagascariensis*, *N. masoalensis*) which precludes any conclusion on their monophyly and their position in the tree.

AFLP data

2697 bands were obtained for 93 taxa but there was a lot of missing data for each pair of primers tested. This probably explains the lack of resolution of the analyses that leads to a poorly resolved tree; this is particularly the case for the deep nodes of the phylogeny (Fig. 2-AFLP). A lot of the more terminal relationships retrieved here were weakly supported with bootstrap values below 0.80. However hybrid taxa grouped together with their respective parent species or other hybrids that share a parent species (*N.* × *trichocarpa* (*N. gracilis* × *N. ampullaria*), *N. ampullaria* × *N. tobaica* within the *N. ampullaria* group; *N. gracilis* × *N. bicalcarata* and *N. bicalcarata* × *N. ampullaria* group with *N. bicalcarata*) which validates our approach and indicates that our analyses do not reflect leaf contamination by fungi for instance. The tree obtained showed poor congruence with ITS and chloroplastic data, few of the previously described geographical clusters were observed. However monophyly and basal position of the western clade were confirmed. We also retrieved similar geographical clustering for species from N/W Sumatra (clade A1), from Borneo (clade B) and from Philippines (clade C1). On the other hand, some results appeared in total contradiction with previous reconstructions, for instance, species of the *hamata* group appeared dispatched throughout the phylogenetic tree and this lack of congruence is discussed below.

Discussion

Molecular phylogeny of the genus

This study results in an updated yet not fully resolved phylogeny of the genus *Nepenthes*. The four additional chloroplastic genes confirm that cpDNA is poorly resolutive for *Nepenthes*. Though we successfully managed to conduct AFLP analyses, this genome wide approach revealed to be poorly resolutive and showed poor congruence with the analyses of chloroplastic and nuclear sequences obtained. Finally, the nuclear marker ITS appeared to be the most promising of the markers tested here to investigate *Nepenthes* molecular phylogeny.

Phylogenetic reconstructions at the infrageneric level are known to be difficult for plants (Despres *et al.*, 2003, Pellmyr *et al.*, 2007), and *Nepenthes* is one of these challenging genera. The lack of congruence between AFLP and chloroplastic DNA analyses could have several explanations. The general lack of resolution of AFLP data suggests that we might have numerous homoplasious characters, i.e. shared bands that do not represent homologous characters but artefacts (Bussell *et al.*, 2005, Althoff *et al.*, 2007). There is also a lot of missing data in this dataset and each pair of primers tested did not work on the same set of species which probably introduced some methodological artefacts in our data. If the species in our sampling show different levels of ploidy (Lowrey & Timothey, 1991, Heubl & Wistuba, 1997), this could also bias our AFLP analyses and for now we have little and discordant information on this issue (see Cheek & Jebb, 2001 for a review). Repeated introgressions could also explain the incongruency between chloroplastic and nuclear DNA analyses. Some species of the genus are commonly observed to grow in sympatry and since physiological barriers to interspecific hybridisation seem weak, they frequently hybridize (Cheek & Jebb, 2001). However, if introgression was the main explanation for incongruency between AFLP data and cp DNA data, we should at least find some congruency between AFLP data and ITS data. The low level of variation in cpDNA and AFLP markers, and their lack of congruence probably reflect both methodological issues concerning the obtention of AFLP data, frequent hybridisation events but also taxonomic issues. Indeed, the definitions of *Nepenthes* species are widely based on morphological characters that might show homoplasy such as pitcher morphology. Whether morphological characters are reliable or not, they are extensively used for *Nepenthes* genus but with contrasted indulgence concerning species boundaries. For instance, on the one hand some “groups or pairs of species are extraordinary difficult to interpret” according to Clarke (Clarke, 2001) and described as close but distinct relatives (e.g. *N. diatas* and *N. densiflora* ; *N. ventricosa* and *N. sibuyanensis*) only on the basis of slight variations in their morphological characters. On the other hand, other taxa such as *N. mirabilis* or *N. rafflesiana* include varieties that conspicuously differ from each other. For instance, in the *N. mirabilis* species, the *echinostoma* variety, endemic to Northern Borneo, produces larger leaves and larger pitchers with a broader and flattened peristome, which makes this taxon far more different than all the other local variants known for this species that has the widest geographic repartition in the genus. *Nepenthes rafflesiana* var. *typica* and var. *elongata* are still considered as varieties, even if they differ in their ecology (Clarke, 1997), pitcher morphology and trapping strategies (Di Giusto *et al.*, 2009, Gaume & Di Giusto, 2009a) and probably also in their floral phenology, architecture and odour cue (unpublished data). Both better resolved phylogenies and population genetics studies, e.g. microsatellites and AFLP, are necessary to clarify species relationship and boundaries in the *Nepenthes* genus.

Finally, the nuclear ITS appears as the most promising markers for investigating *Nepenthes* phylogeny as it gave a relatively well resolved tree. Altogether, the data obtained is a step forward to obtaining a robust and resolved phylogenetic reconstruction for the genus *Nepenthes*. We discuss here its implications for existing taxonomic subdivisions.

Danser's infrageneric subdivisions

Danser established 6 infrageneric clades in his monograph on *Nepenthes* genus: *Vulgatae, Montanae*, *Nobiles*, *Regiae*, *Insignes* and *Urceolatae* for the ~ 60 species he recognized in 1928 (Danser, 1928). As previously evidenced by Meimberg's et *al*. although on a less resolved phylogeny (Meimberg *et al.*, 2001), these groups are not monophyletic here. This indicates that the Danser's morphological and biogeographical based classification does not reflect the phylogenetic history of the genus (see AFLP, cpDNA and ITS trees). Nevertheless, cpDNA tree indicates that almost all the *Regiae* species group in the clade B and the positions of the two remaining *Regiae* (*N. truncata* and *N. boschiana*) are only poorly supported according to pp values. It seems that Danser was also right concerning all the *N. bongso* relatives, retrieved in the clade A and classified by him in the *Montanae* group. Although not all the *Montanae* species group together, most species in this Sumatran clade are indeed closely related.

The Western clade

Both AFLP and chloroplastic data indicate that western species (*N. madagascariensis*, *N. masoalensis*, *N. khasiana* and *N. distillatoria*) group together which independently confirm previous results (Meimberg & Heubl, 2006). Meimberg et *al.* interpretation is that there has been a western colonization of remote islands (Sri Lanka and Madagascar) from a continental ancestor (Meimberg & Heubl, 2006). *Nepenthes pervillei*, an endemic species from the Seychelles, is absent from this study, but previous phylogeny obtained with *trnK* intron seem to confirm this hypothesis, placing it more basally than Malagasian species (Meimberg *et al.*, 2001). Unfortunately, we did not manage to obtain ITS data for the three western species (*N. distillatoria*, *N. madagascariensis*, *N. masoalensis*) of our sampling to confirm their basal position. Establishing biogeographical scenarios will necessitate further analyses based on a more robust phylogeny and up to date methods of biogeographical history (Ree *et al.*, 2005). Based on current results that fact that western species are basal could just reflect that there are clearly differentiated from Asian species. Another interpretation of their basal position could be that they differentiated during the separation of Sri Lanka and Madagascar from continental masses. It is difficult to formally test the different plausible biogeographical scenarios, for now our data do not contradict the scenario put forward by Meimberg and collaborators. But further work needs to focus on obtaining a better resolved phylogeny that could also be calibrated.

The North/Central Sumatra clade

Species from North Sumatra form a monophyletic clade according to ITS, and cpDNA data also cluster most species from Sumatra together. The N/W Sumatra region that encompasses Aceh, Lake Toba and the Malaisian state of Burat is considered as a centre of diversity of the *Nepenthes* genus. There, species with a very limited distribution (e. g. *N. talagensis*) occur with others exhibiting a wide geographical distribution and great morphological variations (e. g. *N. bongso*) (Clarke, 2001, McPherson, 2009). Our chloroplastic data indicate that *N. bongso* and *N. talagensis* are closely related and may even be the same species (Nerz & Wistuba, 1994, Clarke, 2001). Even if closely related, our two *N. bongso* (ex *N. carunculata*) specimens group together but in a clade separated from *N. bongso*. The question of the occurrence of infraspecific taxa within *N. bongso* may thus be not as definitive as reported by McPherson (2009) and this species may not be as closely related to *N. ovata* and *N. densiflora* as reported by Clarke (2001). Molecular data confirm that former Salmon’s and Maulder’s *N. xiphioides* and Cheek and Jebb’s *N. pectinata* are closely related if not synonymous. These two taxa, themselves potentially synonyms of *N. gymnamphora* (Clarke, 2001, McPherson, 2009), do not group with *N. gymnamphora* on the cpDNA tree but are confirmed to be - at least - closely related. Both nuclear and chloroplastic sequences confirm the view of Cheek and Jebb and the cladistic analysis of Clarke that *N. densiflora* and *N. diatas* are closely related (Cheek & Jebb, 2001, Clarke, 2001). In their revision of the genus, Cheek and Jebb treat *N. longifolia* and *N. sumatrana* as synonyms whereas Clarke, also basing its conclusions on morphological characters, treats Nerz’s and Wistuba’s *N. longifolia* as a different species than *N. sumatrana* (Nerz & Wistuba, 1994, Cheek & Jebb, 2001, Clarke, 2001). Neither AFLP data nor cpDNA (ITS sequence not obtained) grouped these two species together and this is also the case of the phylogenies previously published (Meimberg *et al.*, 2001, Meimberg & Heubl, 2006). If *N. sumatrana* and *N. longifolia* thus belong to a clade different from the clade of *N. rafflesiana*, this would represent a conspicuous example of parallel evolution of pitcher morphologies and trapping mechanisms. *N. rafflesiana* var. *typica* and *N. rafflesiana* var. *elongata* have been shown to have very different pitcher morphologies and associated trapping strategies that are caused by the loss in the var. *typica* of the waxy layer (Di Giusto *et al.*, 2009, Gaume & Di Giusto, 2009a). Similarly, *N. sumatrana* has infundibulate (funnel-shaped) upper pitchers that lack a waxy zone whereas *N. longifolia* have very different elongate upper pitchers whose inner walls are covered with a waxy layer (Clarke, 2001). These taxa could be the Sumatran counterparts of *Nepenthes rafflesiana* var. *typica* and var. *elongata*, respectively.

The Bornean clade

The phylogenies obtained reveal a second clade, highly sustained in the cpDNA tree as well as with ITS sequences for the four species successfully sequenced in this clade which encompasses some of the Bornean species (Node B – Fig. 2-cpDNA,ITS). Our reconstruction confirms the view of Cheek and Jebb who suggest that *N. fusca, N. faizaliana, N. pilosa, N. stenophylla* and *N. veitchii* are closely related (Cheek & Jebb, 2001). Our data also confirm the clustering of *N. maxima* with *N. burdbidgeae* made by these authors (Cheek & Jebb, 2001). Two out of the three species with "toilet-seat"-shaped pitchers that have been shown to derivate nutrients from vertebrate faeces (*N. macrophylla* and *N. rajah*) group together in the phylogeny based on cpDNA. Unfortunately, we did not obtain any ITS and AFLP data to confirm this result. The position of this clade is poorly supported in the cpDNA, it may likely be integrated in the Bornean clade in further phylogenies.

Conclusion and Perspectives

Our results indicate that the *Nepenthes* phylogeny shows some geographical structures in some parts of the tree and that previous taxonomic subdivisions are partly invalidated by molecular data. The global lack of resolution of the various markers investigated and their lack of congruence make the phylogenetic reconstruction a difficult task. These difficulties could be due to some methodological problems concerning the obtention of AFLP data, a global low level of evolution in chloroplastic DNA which may be due partly be to a rapid diversification of the genus. The completion of the ITS dataset and the addition other nuclear markers (such as ETS that has proved useful as an additional source of information to ITS in plant phylogenies) might lead to a more robust reconstruction. This would allow testing biogeographical hypotheses that are difficult to establish for the moment. The geographic patterns obtained here (clustering of some species according to their geographical proximity but also the occurrence of some species that are dispatched more haphazardly in the phylogenetic tree) suggest some promising perspectives for testing the role of refuge and/or island formation on speciation in this area which has a very eventful geological history with important variations of sea level and climate changes in the Pleistocene (Hall, 1998). The phylogenies obtained here are a step forward this goal. Eighty years later, Danser's view is still topical: "The combination of the sharp limitation externally and the difficulty of an internal division result in making *Nepenthes* one of the most natural groups of the vegetable kingdom" (Danser, 1928).

Tables

Species	**Geographic origin**	**ITS**	**rps16**	**trnL**	**trnD**	**ycf6**	**AFLP**	**Danser, 1928**
N. adnata Tamin & M. Hotta ex Schlauer	S	x	x	x	x	x		
N. alata var. *typica* Blanco	P	x	x	x	x	x	x	Vulgatae
N. albomarginata T. Lobb ex Lindl.	B, PM, S		x	x	x	x		Vulgatae
N. ampullaria × *N. tobaica*							x	Urceolatae
N. ampullaria 'black' Jack	B, S, PM, Th, NG		x	x	x	x		Urceolatae
N. ampullaria 'green' Jack	–		x	x	x	x	x	Urceolatae
N. ampullaria 'red' Jack	–				x	x		Urceolatae
N. ampullaria 'tricolor' Jack	–							Urceolatae
N. ampullaria 'typica' Jack	–		x	x	x	x	x	Urceolatae
N. argentii Jebb & Cheek	P		x	x	x	x	x	
N. aristolochioides Jebb & Cheek	S				x	x	x	
N. belli K. Kondo	P	x	x	x	x	x	x	
N. bicalcarata × *N. ampullaria*							x	
N. bicalcarata Hook. f.	B		x	x	x	x	x	Urceolatae
N. bongso (*N. carunculata*) Danser Korth.	S		x	x	x			Montanae
N. bongso (*N. carunculata*) Danser Korth.	S	x	x	x	x	x	x	
N. bongso (*N. talagensis*) Nerz & Wistuba Korth.	S		x	x	x	x		
N. bongso '*sumatra'* Korth.	S	x	x	x	x	x	x	
N. boschiana Korth.	B	x	x	x	x	x	x	Regiae
N. burbigdae Hook. f. ex Burb.	B	x	x	x	x	x	x	Regiae
N. burkei Mast.	P	x	x	x	x	x	x	Insignes
N. campanulata Sh. Kurata	B		x	x			x	
N. clipeata Danser	B		x	x	x	x	x	
N. copelandii Merr. ex Macfarlane	P	x	x	x		x	x	
N. danseri Jebb & Cheek	NG		x	x	x	x	x	
N. densiflora Danser	S	x	x	x	x	x	x	
N. diatas Jebb & Cheek	S	x	x	x	x	x	x	
N. distillatoria L.	Sri Lanka		x		x	x		Vulgatae
N. dormoniana JJ							x	
N. dubia (*N. tenuis*) Danser	S		x	x	x	x	x	Montanae
N. ephippiata Danser	B	x	x	x	x	x	x	Regiae
N. eustachya Miq.	S	x	x	x	x	x	x	
N. eymae Sh. Kurata	Sul.		x	x	x	x		
N. faizaliana J. H. Adam & Wilcock	B		x	x	x	x	x	
N. fusca Danser	B	x	x	x	x	x	x	Regiae
N. glabrata J. R. Turnbull & A. T. Middleton	Sul.	x	x	x	x	x	x	
N. gracilis × *N. bicalcarata*							x	Vulgatae
N. gracilis 'red' Korth.	B, S, PM, Sul, Th.	x	x	x	x	x		Vulgatae
N. gracilis 'typica' Korth.	–		x	x	x	x	x	Vulgatae
N. gracillima × *N. ventricosa*			x	x	x	x		
N. gracillima Ridl.	PM		x	x	x	x		Montanae
N. gymnamphora (ex. *N. pectinata*) Nees	S		x	x	x	x		Montanae
N. gymnamphora Nees	Java, S		x	x	x	x	x	Montanae
N. gymnamphora Nees (*N. xiphioides*)	–		x	x	x	x	x	Montanae
N. hamata J. R. Turnbull & A. T. Middleton	Sul.	x	x	x	x	x	x	
N. hirsuta Hook. f.	B		x	x	x	x	x	Nobiles

N. hispida Beck	B		x	x	x		x	
N. inermis Danser	S	x	x	x	x	x	x	Montanae
N. insignis Danser	NG	x	x	x	x	x	x	Insignes
N. izumiae Troy Davis, C. Clarke & Tamin	S	x	x	x	x	x	x	
N. jacquelinae C. Clarke, T. Davis & Tamin	S	x	x		x	x		
N. khasiana Hook. f.	Indian Khasi	x	x	x	x	x	x	Vulgatae
N. lamii (ex. *N. vieillardii*) Jebb & Cheek	NG				x		x	
N. lowii Hook. f.	B		x	x	x	x	x	Regiae
N. macfarlanei Hemsl.	PM	x	x	x	x	x	x	Montanae
N. macrophylla (Marabini) Jebb & Cheek	B		x		x	x	x	
N. macrovulgaris J. R. Turnbull & A. T. Middleton	B		x	x	x	x	x	
N. madagascariensis Poir.	Madagascar	x	x	x	x	x	x	Vulgatae
N. mapuluensis J. H. Adam & Wilcock	B						x	
N. masoalensis Schmid-Hollinger	Madagascar		x	x	x	x	x	
N. maxima Reinw. ex Nees	Sul.	x	x	x	x		x	Regiae
N. merilliana Macfarl.	Ph.	x	x	x	x	x	x	Insignes
N. mikei Salmon & Maulder	S		x	x	x	x	x	
N. mindanaonensis Sh. Kurata	Ph.	x	x	x	x	x	x	
N. mira Jebb & Cheek	Ph.	x	x	x	x	x	x	
N. mirabilis 'Hong-Kong' (Lour.) Druce	SE Asia, Aust.	x	x	x	x	x		Vulgatae
N. mirabilis var. *anamensis* Macfarl. (Lour.) Druce	–		x	x	x	x	x	Vulgatae
N. mirabilis var. *distillatoria* (Lour.) Druce	–		x	x	x	x		Vulgatae
N. mirabilis var. *echinostoma* (Lour.) Druce	–		x	x	x	x	x	Vulgatae
N. mirabilis var. *globosa* (Lour.) Druce	–						x	Vulgatae
N. mirabilis var. *typica* (Lour.) Druce	–	x	x	x	x	x	x	Vulgatae
N. mirabilis '*winged*' (Lour.) Druce	–		x	x	x	x	x	Vulgatae
N. muluensis M. Hotta	B	x	x	x	x	x	x	
N. murudensis Culham ex Jebb & Cheek	B	x						
N. neoguineensis Macfarl.	NG		x		x	x	x	Vulgatae
N. northiana Hook. f.	B	x	x	x	x	x		Insignes
N. ovata Nerz & Wistuba	S	x	x	x	x	x	x	
N. petiolata Danser	Ph.	x	x	x	x	x	x	Insignes
N. philippinensis Macfarl.	Ph.	x	x	x	x	x	x	Vulgatae
N. pilosa Danser	B	x	x	x	x	x	x	Regiae
N. rafflesiana × *N. hirsuta*							x	Insignes
N. rafflesiana var. *elongata* Hort.	B, PM, S		x	x	x	x		Insignes
N. rafflesiana var. *gigantea*	–		x	x	x	x	x	Insignes
N. rafflesiana var. *nivea* Hook. f.	–		x	x	x	x	x	Insignes
N. rafflesiana var. *typica* Jack	–		x	x	x	x		Insignes
N. rajah Hook. f.	B		x	x	x	x	x	Regiae
N. ramispina (*N. gracillima*) Ridl.	PM		x	x	x	x		
N. ramispina Ridl.	PM	x	x	x	x	x	x	
N. reindwartiana Miq.	B, S		x	x	x	x	x	Vulgatae
N. rhombicaulis Sh. Kurata	S	x	x	x	x	x	x	
N. sanguinea Lindl.	PM, Th.		x	x	x	x		Montanae
N. sibuyanensis Nerz	Ph.	x	x	x	x	x	x	
N. singalana Becc.	S		x				x	Montanae
N. spathulata Danser	S	x	x	x	x			
N. spectabilis Danser	S	x	x	x	x	x	x	Nobiles
N. stenophylla Mast.	S	x	x	x	x	x	x	Regiae

N. sumatrana 'green leaves' (Miq.) Beck	S	x	x	x	x	x		
N. sumatrana 'red leaves' (Miq.) Beck	S		x	x		x	x	
N. talagensis Nerz & Wistuba	B, Sul.	x	x	x	x	x	x	
N. tentaculata Hook. f.	B, Sul.	x	x	x	x	x		Vulgatae
N. tenuis Nerz & Wistuba	S				x	x	x	
N. thorelii Lecomte	PM	x	x	x	x	x		
N. tobaica Danser	S	x	x	x	x	x	x	Vulgatae
N. tomoriana Danser	Sul.		x	x	x	x	x	Vulgatae
N. truncata Macfarl.	Ph.	x	x	x	x	x	x	Regiae
N. veitchii Hook. f.	B		x	x	x	x	x	Regiae
N. ventricosa Blanco	Ph.		x	x	x	x	x	Insignes
N. villosa Hook. f.	B	x	x	x	x	x	x	Insignes
N. vogelii Schuit. & de Vogel	B	x	x	x	x	x	x	
N. × *trichocarpa* Miq.			x	x		x	x	
N. × *hookeriana* Lindl.			x	x		x	x	
Drosophyllum lusitanicum L.		x	x	x			x	
Drosera sp. L.		x		x		x	x	
Drosera sp. L.		x		x		x		

Table 1 : List of *Nepenthes* taxa, outgroups and markers investigated in this study. The geographical origin is provided : Aust. = Australia ; B : Borneo ; NG = Papua/New-Guinea ; Ph. = Philippines ; PM = Malay Peninsula ; S = Sumatra ; Sul. = Sulawesi ; Th ; = Thailand + Cambodge. The names put within brackets are synonyms. All samples have been provided by J.-J. Labat.

Primer	**5' sequence 3'**	***T_m* (°C) and elongation time**
ITS1	GAA GGA GAA GTC GTA ACA AGG	58
ITS4	CCT CCG CTT ATT GAT ATG C	1min
rps16F	GGT AGA AAG CAA CGT GCG ACT T	50
rps16R2	CGG GAT CGA ACA TCA ATT GCA AC	1min
trnLF-R	TGC CAG GAA CCA CAT TTG AAC T	54
trnLF-F	TCC GTC GAC TTT ATA AGT TGT G	1min
trnD^{GUC}-F	ACC AAT TGA ACT ACA ATC CC	48
trnT^{GGU}-R	CTA CCA CTG AGT TAA AAG GG	1min30sec
psbM-R	CCA AGT TAG TTA ATG AAG AA	58
ycf6-F	ATG GAT ATA GTA AGT CTY GCT TGG GC	1min20sec
trnCR	CAC CCR GAT TYG AAC TGG GG	48
rpoB	CKA CAA AAY CCY TCR AAT TG	1min

Table 2: List of primers and T_m.used for DNA amplification and sequencing.

Figures

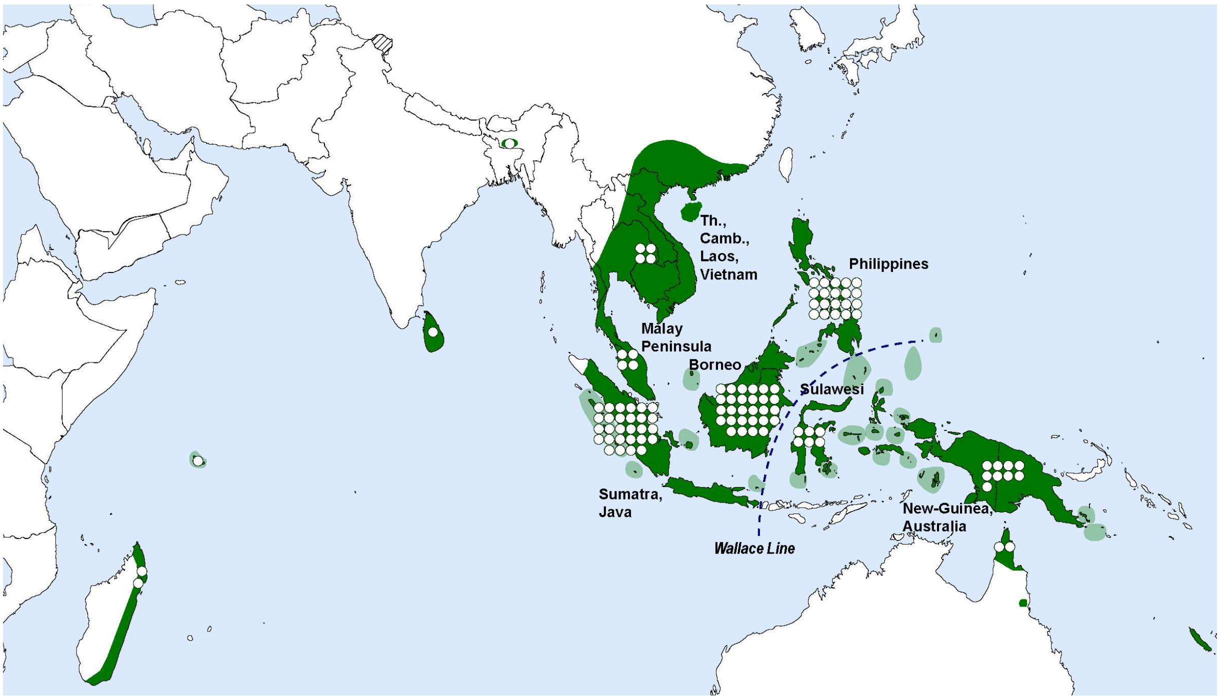
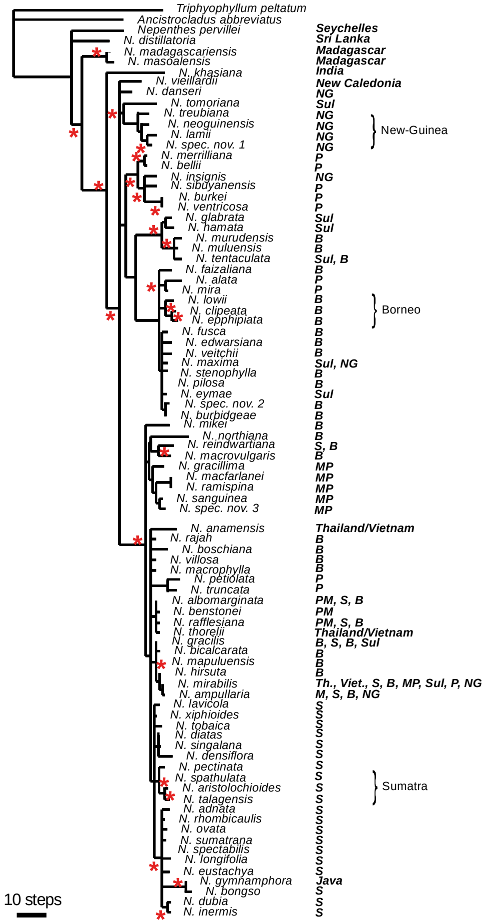


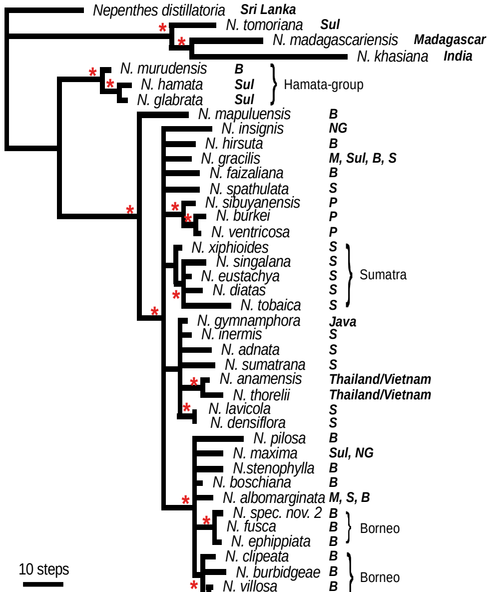
Figure 1: Geographical distribution of *Nepenthes*. Dots indicate endemic species for the eight geographic groups retained, which names are indicated on the map, plus a western group gathering Indian Khasi, Sri Lanka and Seychelles.

Figure 2 (following page) : **trnK** and **PTR1** trees (chloroplastic and nuclear sequences respectively) are redrawn from Meimberg *et al.* (2006). Stars indicate nodes with bootstrap values > 70. **AFLP**, **cpDNA** and **ITS** trees obtained here,. The geographic distribution has been indicated on each branch and , Danser's infrageneric classification is indicated in front of each species name. Letters and arrows indicated the main clades that are discussed in the text.

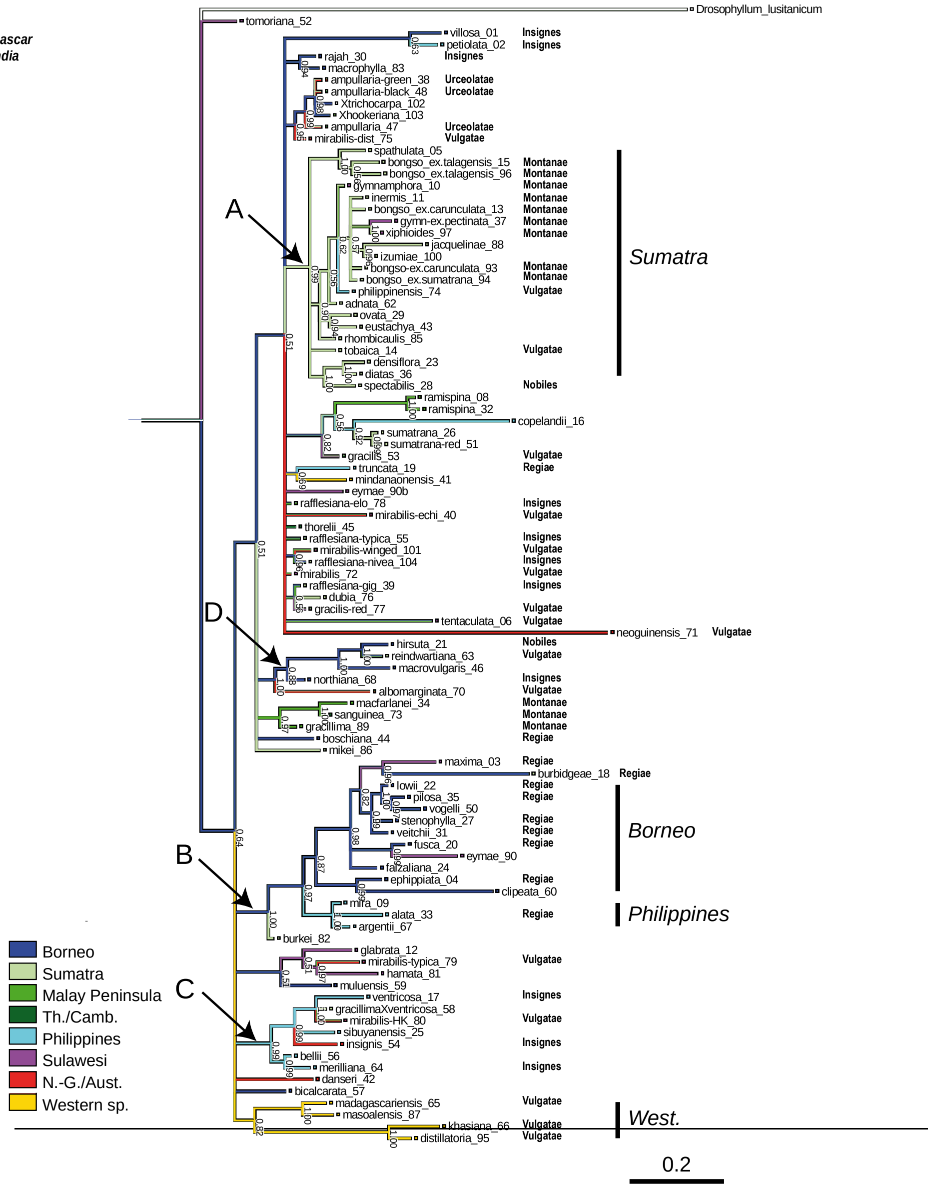
trnK

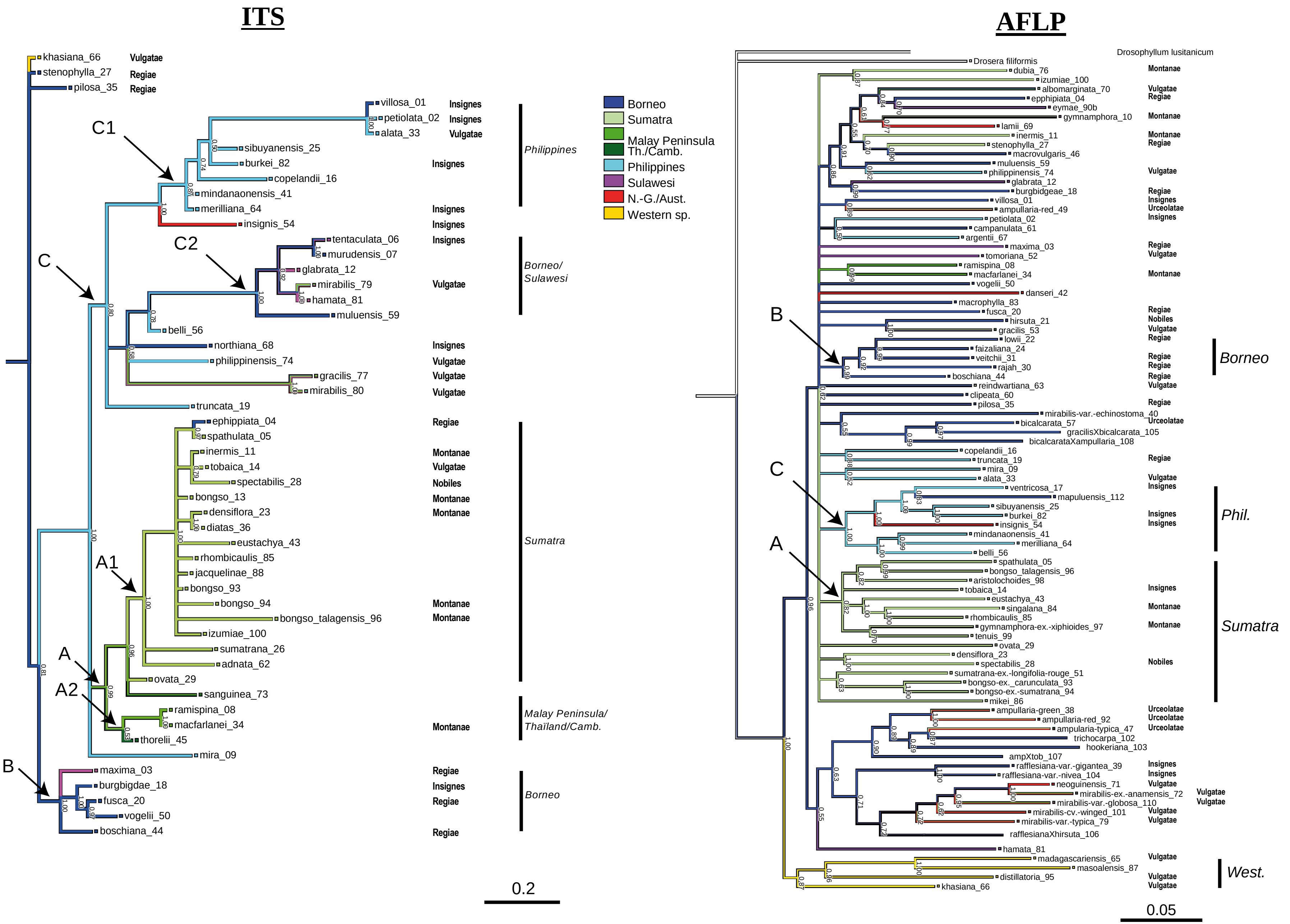


PTR1



cpDNA




ITS
AFLP
Borneo
Sumatra
Malay Peninsula Th./Camb.
Philippines
Sulawesi
N.-G./Aust.
Western sp.
khasiana_66
stenophylla_27
pilosa_35
villosa_01
petiolata_02
alata_33
sibuyanensis_25
burkei_82
copelandii_16
mindanaonensis_41
merilliana_64
insignis_54
tentaculata_06
murudensis_07
glabrata_12
mirabilis_79
hamata_81
muluensis_59
belli_56
northiana_68
philippinensis_74
gracilis_77
mirabilis_80
truncata_19
ephippiata_04
spathulata_05
inermis_11
tobaica_14
spectabilis_28
bongso_13
densiflora_23
diatas_36
eustachya_43
rhombicaulis_85
jacquelinae_88
bongso_93
bongso_94
bongso_talagensis_96
izumiae_100
sumatrana_26
adnata_62
ovata_29
sanguinea_73
ramispina_08
macfarlanei_34
thorelii_45
mira_09
maxima_03
burgbigdae_18
fusca_20
vogelii_50
boschiana_44
C1
C2
C
A1
A
A2
B
Philippines
Borneo/Sulawesi
Sumatra
Malay Peninsula/Thaïland/Camb.
Borneo
0.2
Drosophyllum lusitanicum
Drosera filiformis
dubia_76
izumiae_100
albomarginata_70
epphipiata_04
eymae_90b
gymnamphora_10
lamii_69
inermis_11
stenophylla_27
macrovulgaris_46
muluensis_59
philippinensis_74
glabrata_12
burgbidgeae_18
villosa_01
ampullaria-red_49
petiolata_02
campanulata_61
argentii_67
maxima_03
tomoriana_52
ramispina_08
macfarlanei_34
vogelii_50
danseri_42
macrophylla_83
fusca_20
hirsuta_21
gracilis_53
lowii_22
faizaliana_24
veitchii_31
rajah_30
boschiana_44
reindwartiana_63
clipeata_60
pilosa_35
mirabilis-var.-echinostoma_40
bicalcarata_57
gracilisXbicalcarata_105
bicalcarataXampullaria_108
copelandii_16
truncata_19
mira_09
alata_33
ventricosa_17
mapuluensis_112
sibuyanensis_25
burkei_82
insignis_54
mindanaonensis_41
merilliana_64
belli_56
spathulata_05
bongso_talagensis_96
aristolochoides_98
tobaica_14
eustachya_43
singalana_84
rhombicaulis_85
gymnamphora-ex.-xiphioides_97
tenuis_99
ovata_29
densiflora_23
spectabilis_28
sumatrana-ex.-longifolia-rouge_51
bongso-ex._carunculata_93
bongso-ex.-sumatrana_94
mikei_86
ampullaria-green_38
ampullaria-red_92
ampularia-typica_47
trichocarpa_102
hookeriana_103
ampXtob_107
rafflesiana-var.-gigantea_39
rafflesiana-var.-nivea_104
neoguinensis_71
mirabilis-ex.-anamensis_72
mirabilis-var.-globosa_110
mirabilis-cv.-winged_101
mirabilis-var.-typica_79
rafflesianaXhirsuta_106
hamata_81
madagascariensis_65
masoalensis_87
distillatoria_95
khasiana_66
B
C
A
Borneo
Phil.
Sumatra
West.
0.05
Vulgatae
Regiae
Insignes
Urceolatae
Montanae
Nobiles

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Chapitre II.2

ORIGINE ET ÉVOLUTION DES MÉCANISMES DE PIÉGEAGE

Ce **Manuscrit II.2** retrace sur la phylogénie obtenue l'évolution des caractères « présence d'une zone cireuse » et « d'un liquide viscoélastique » obtenus pour la majorité des espèces et pour les urnes juvéniles et terrestres. Le caractère « liquide viscoélastique » correspond-t-il à une acquisition au cours de la diversification du genre *Nepenthes* ? Si non, est-il plésiomorphe et homologue au mucilage de *Drosera* et *Drosophyllum* et a-t-il été perdu à une ou de multiples reprises au sein du genre ? Au sein du complexe *Nepenthes rafflesiana*, la variété *typica* perd sa zone cireuse lors de l'acquisition de la lianescence, la variété *gigantea* bien plus tôt dans son développement, alors que la variété *elongata* possède une zone cireuse y compris au niveau de ses urnes aériennes (Di Giusto *et al.*, 2009, Gaume & Di Giusto, 2009a). Que nous apprennent les toutes premières urnes produites sur l'évolution des systèmes de piégeage du genre ? De telles modifications du développement sont-elles un cas isolé au sein du complexe *Nepenthes rafflesiana* ou préfigurent-elles un mécanisme evo-devo général au sein du genre ?

Bonhomme, V., Gaume, L. & Jousselin, E. (in prep.) Origin and evolution of the carnivorous traits in the genus *Nepenthes*.

ORIGIN AND EVOLUTION OF CARNIVOROUS TRAITS IN THE GENUS *NEPENTHES*

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Introduction

Molecular phylogenies have brought to contemporary biology a unique tool to infer diversification patterns as well as the opportunity to test hypotheses concerning the issue of how ecological mechanisms drive these patterns.

Carnivorous plants circumvent shortage of nutrients in the environments they colonize by obtaining nutrients through an array of foliar adaptations associated with the attraction, the capture and the digestion of arthropods (Juniper *et al.*, 1989). Carnivory appeared independently at least 6 times in Angiosperms and is represented today by a diversity of specialized forms and trapping strategies displayed in 18 genera. Studies on carnivorous plants are mainly focused on the mechanisms of the trapping systems in single species or in few species. Some studies nevertheless attempt to embrace the evolution of carnivorous traits in ecological and phylogenetic contexts. For instance Fleishmann *et al.* (2010) proposed an integrative study on the biogeographical history of the neotropical genus Genlisea (Lentibulariceae) and a reconstruction of floral and carnivorous traits on a well-resolved phylogeny of the genus (Fleischmann *et al.*, 2010). In the Caryophyllales order rich in carnivorous species, Gibson and Waller (2009) modeled the evolutionary steps from the *Drosera*-like flypaper traps to the *Dionae* / *Aldrovanda* snap-traps, whose evolution has likely been driven by selection towards the capture of larger prey items (Gibson & Waller, 2009). Earlier, Heubl *et al.* (2006) have revisited the functional evolution in Caryophyllales and proposed an evolutionary pathway from glandular surfaces to flypaper traps, snap traps, pitchers and also towards species that have secondary lost their carnivorous habits (Heubl *et al.*, 2006).

Our study is focused on the evolution of some carnivorous characters of the Caryophyllale genus of climbing pitcher-plants, *Nepenthes*. This is one of the most species-rich carnivorous genus with at least 120 species, mainly distributed in SE Asia (McPherson, 2009). Their traps are leaves modified as pitchers that rely on slippery surfaces and sticky fluids to capture their prey. These pitcher traps typically have three functional features (Figure 1). First, the ridged peristome that borders the mouth of the pitcher is involved in insect fall through its anisotropic and wettable structure (Bohn & Federle, 2004), the production of floral odors (Di Giusto *et al.*, 2010) and the nectar which favors humidity condensation and insect aquaplaning (Bauer *et al.*, 2008). The peristome is separated from the digestive part of the pitcher by a conductive zone whose inner walls are covered in most of the species by a waxy layer mainly composed of very-long-chain aldehydes (Riedel *et al.*, 2003). This waxy zone is an important retentive device (Juniper & Burras, 1962, Gaume *et al.*, 2002, Gaume & Di Giusto, 2009a). The combination of reverse anchorage sites (Gaume et al. 2002), wax roughness (Gorb et al. 2005), fragility of individual wax platelets (Juniper and Buras 1962, Gaume et al. 2004, Riedel et al. 2007), wax chemical interaction with insect's adhesive micro-secretions (Gaume et al. 2004) cause insect fall and retention within the pitcher. Below, the basal and digestive zone is filled with an acidic liquid, the enzymes of which (Jentsch, 1972, Hatano & Hamada, 2008) and the infauna it hosts (Clarke & Kitching, 1993) achieve the exodigestion of prey. Besides digestion, this fluid has been shown to be involved in retention, first in the species *N. rafflesiana* (Gaume & Forterre, 2007, Di Giusto *et al.*, 2008), then in numerous other species (Bonhomme *et al.*, submitted to *Functional Ecology*) distantly related within the genus (Bonhomme *et al.*, in prep.-a, Bonhomme *et al.*, in prep.-c).

One of the most intriguing aspects of the biology of the genus *Nepenthes* and what makes it a four-star model for evolutionary biologists is that both the combination of these devices and the morphology of the pitchers vary between species and throughout ontogeny. For instance, the combination of wax density and viscoelasticity of the digestive liquid on a representative subset of species has been shown to greatly vary between species (Bonhomme *et al.*, in prep.-c). The species with a very viscoelastic liquid were not very waxy and *vice-versa*. Viscoelasticity of the digestive fluid was shown to be very efficient at trapping both flies and ants, whereas, wax was only efficient at trapping ants. This suggests that the entomofauna present in the plant’s habitat could be an important selective pressure on its trapping strategy. Some outlier strategies also exist in the genus: the non-waxy and non-viscoelastic *N. ampullaria* is partly detritivorous (Moran *et al.*, 2003); three Bornean montane species have “toilet-seat”-shaped pitchers that lack a waxy layer and attract tree-shrews, assimilating their faeces (Chin *et al.*, 2010); the non-waxy and non-viscoelastic *N. bicalcarata* uses its symbiotic ant to trap prey (Bonhomme *et al.*, 2010).

In addition to these interspecific differences, there is also a level of intra-specific variation: some species present a morphological and functional pitcher dimorphism throughout the plant’s ontogeny (Moran, 1996, Di Giusto *et al.*, 2009). This is common in climbing plants (Putz & Mooney, 1991b) and general in *Nepenthes* pitcher plants. Lower (or terrestrial) pitchers whose apertures are directed towards the plant axis are produced during the self-supporting stage and upper (or aerial) pitchers start to differentiate when the plant enters into its climbing phase. The aperture of these upper pitchers is generally directed outwards and the tendrils bearing them are often twisted around a vegetal support (Figure 1). In *N. rafflesiana* var. *typica*, the pitcher dimorphism is coupled with a modification of its trapping mechanism. Its lower pitchers bear a waxy layer which is progressively lost when the plant ages and starts to produce upper pitchers (Gaume & Di Giusto, 2009a). Interestingly these waxy-lower pitchers mainly trap ants (Gaume & Di Giusto, 2009a) while the non-waxy but highly viscoelastic upper pitchers have a more diversified prey spectra including a number of flying insects (Di Giusto *et al.*, 2008). By contrast, the *elongata* variety of *N. rafflesiana* keeps the waxy layer throughout plant ontogeny and mainly captures ants (Gaume & Di Giusto, 2009a).

In *Nepenthes*, the exhibition of outlier trapping strategies, as described above, with peculiar pitcher morphology (Cresswell, 1998) and absence of typical retentive devices is likely to be linked to the partly detritivorous or coprophagous habits of the plant species (Moran *et al.* 2003; Clarke *et al.* 2009; Lee *et al.* 2010). But we do not know for the vast majority of the strictly carnivorous species how the two typical (Bonhomme *et al.*, submitted to *Functional Ecology*) retentive devices, the waxy layer and the viscoelastic fluid, are distributed throughout the *Nepenthes* phylogeny and why we observe such a diversity. Gaume and Di Giusto (2009) suggested that the waxy layer could be an ancestral character and that several species have lost the waxy layer at the benefit of a viscoelastic fluid (Gaume & Di Giusto, 2009b). They also suggested that the evolution of new pitcher forms such as the infundibular (funnel-shaped) form could be linked to the developmental deletion of the waxy zone. This study investigates the evolutionary history of these two retentive traits (wax and viscoelasticity) and the diversification of *Nepenthes'* trapping strategy throughout a molecular phylogeny of the genus. By mapping these traits onto the phylogeny we addressed several questions. 1) What are the ancestral character states in the genus and how many times have they been acquired and lost? 2) Is wax lost during the development, i.e. do plants that have juvenile pitchers with wax necessarily have waxy adult pitchers? And if not, did these losses occur repeatedly during evolution? 3) Is the acquisition of a viscoelatic digestive liquid associated with the loss of wax? 4) Are acquisitions and losses of wax and viscoelasticity associated with changes in pitcher morphology? 5) Is the evolution of these characters (wax and viscoelasticity) associated with peculiar environmental conditions (e.g. altitudinal range of species)?

Materials and Methods

Measurements of the viscoelasticity of digestive fluids

The measurements of viscoelasticity (VE) have been carried out in July 2009 and July 2010 in Peyrusse-Massas (France) on the J.-J. Labat's collection recognized in 1995 as the National French Conservatory of Carnivorous Plants. The liquid of 75 *Nepenthes* species have been sampled on lower pitchers (their opening dating approximately from one week). Upper pitchers produced when the plants enter their climbing phase were rarely available in the greenhouse but when they were present, the viscoelastic status (viscoelastic or not) of their liquid was similar to that obtained for the lower pitchers.

The viscoelasticity of the mucilage of *Drosera adelae*, *Drosera madagascariensis*, *Drosera rotundifolia* and *Drosophyllum lusitanicum* was also measured. The latter species belong to two genera closely related to *Nepenthes*, the genus *Drosera* and the monotypic genus *Drosophyllum*. Since their leaves produce very little but very sticky mucilage, their mucilage was diluted 10 times in water. For these outgroups, our aim was to test if the mucilage has a viscoelastic behavior and not to get quantitative measures.

The viscoelastic behavior of each liquid was investigated with the capillary break-up technique described in Gaume & Forterre (2007) that uses the thinning dynamics of a fluid drop stretched between two plots. All experiments have been made in homogeneous temperature and hygrometry conditions (26-28 °C; 60-70%). The recordings were then analyzed using an ImageJ-R script (Abramoff *et al.*, 2004, R Development Core Team, 2009) that we developed for this purpose. Each fluid was tested three times and mean relaxation times were calculated as an estimation of the fluid viscoelasticity. For water, the non-viscoelastic fluid of reference, we measured six times the capillary pinch-off time, i.e the shortest breaking time for a filament. All measurements were performed under homogeneous temperature conditions. The relaxation times measured for the digestive fluids were compared using Student's *t*-test with the capillary pinch-off time of water. A fluid was conservatively qualified as viscoelastic, if its relaxation time was significantly longer than the capillary pinch-off time of water with $P < 0.01$.

Census of the altitudinal range, pitcher morphologies and presence of wax among Nepenthes *species and seedlings*

We retrieved for the 120 species described in the latest review on the genus made by McPherson (McPherson, 2009), the altitudinal range, the presence of a waxy layer and the pitcher morphology for lower and upper pitchers. For Bornean and Sumatran species, information was compared with that available in the books of Clarke (Clarke, 1997, Clarke, 2001).

The altitudinal range of these species was noted and we categorized their lowland/montane habit with a 1000 m a.s.l. threshold.

The presence of a waxy layer was recorded for upper and lower pitchers on the basis of the photographs available in the book of McPherson (McPherson 2009). The waxy layer presents a white and matt aspect which can unambiguously be identified from photographs. McPherson recognizes seven different pitcher morphologies (‘amphora-shaped/obconic’, ‘cylindrical’, ‘ellipsoidal’, ‘globose’, ‘infundibular’, ‘ovate’ and ‘urceolate’) but with some overlapping in his species description. We chose to merge amphora-shaped/obconic and ovate in an ‘ovate’ category and we retained six other pitcher shapes that in our point of view better discriminate pitcher shapes: ‘ovate-cylindrical’, ‘globose-cylindrical’, ‘cylindrical’, ellipsoidal’, ‘infundibular’, ‘trumpet-like’, ‘globose-infundibular’, ‘trumpet’ and ‘infundibular-cylindrical’ (Table 1, Figure 2).

Thirteen species were discarded from the analyses: *Nepenthes junghunii* (lack of reliable information), *N. mantalingajanensis* (possibly a synonym of *N. mollis* and for both of them: lack of information), *N. mapuluensis* and *N. hirsuta* (incongruency between McPherson and Clarke), *N. paniculata* and *N. rhombicaulis* (lack of information for respectively lower and upper pitchers), *N.*

vieillardii (ambiguous information for upper pitchers), *N. thorelli* (possibly a synonym of *N. smilesii*), *N. beccariana* (possibly a synonym of *N. longifolia*), *N. argentii* that does not appear to have a climbing stage), *N. aristolochoides* and *N. klossi* whose pitcher morphologies are very different than the other species of the genus and would have formed a two-species class.

Molecular phylogeny, reconstruction of ancestral character states and test of correlated evolution

To infer character evolution, we used the molecular phylogeny based on ITS DNA data (Bonhomme *et al.*, in prep.-a) as it was better resolved than reconstructions based on other DNA markers. The fluid-viscoelastic character and the presence of the waxy layer in upper pitchers and lower pitchers were reconstructed using the Maximum Parsimony (MP) optimization criteria implemented in Mesquite (Maddison & Maddison, 2010) on the Bayesian ITS tree. We also reconstructed the evolution of lower and upper pitchers morphology. As discussed in the result section, we reduced the pitcher morphologies to three classes, narrow (ovate-cylindrical, globose-cylindrical, ovate, cylindrical), infundibular (infundibular, globose-infundibular, trumpet) and intermediate. Infundibular-cylindrical and elliptic classes have been discarded from some of the analyses (see Results) as they can be considered as an intermediate in both forms and function, they account for 15 (for lower pitchers) and 16 (for upper pitchers) species out of 107.

These reconstructions first allowed the determination of ancestral character states (for VE and wax), their number of losses and acquisitions as well as investigation of the number of times wax has been lost and pitcher morphology has changed during the transitions from lower to upper pitcher. We then tested for several associations between pitcher traits. We tested whether viscoelasticity and presence of wax on upper pitchers were negatively associated with each other. Further, we also investigated whether different pitcher forms (in lower and upper pitchers) were associated with either wax or viscoelasticity and whether wax losses during the transitions from lower to upper pitcher were associated with changes in pitcher morphology. We also tested whether the sticky and the waxy strategy were each associated with different ecological conditions by testing associations between their presence and the altitudinal range of each species.

We thus conducted a formal test of correlated evolution (Pagel 1994) between all these characters. These correlations were investigated using a likelihood ratio test designed specifically for analysing relationships between two binary characters in a phylogenetic context (Pagel 1994). This is achieved by comparing the fit of two models to the data mapped onto the Bayesian ITS tree in Figures 3 and 4 (Pagel, 1994). For each pair of characters tested, two models of evolution are considered. In one model, two traits are allowed to evolve independently, in the other; they evolve in a correlated fashion. The hypothesis of correlated change is accepted if the dependent model fits the data better than the model of independent change. Significance is assessed through a Likelihood Ratio (LR) test. A Monte Carlo simulation is conducted to find the distribution of the LR under the null hypothesis (independent change) in order to find the *P* value of the LR observed for the real data set (Pagel, 1994). These tests required to prune the species from the tree for which we lack information on one or another character and to solve polytomies by arbitrarily fixing a very low branch length to unresolved nodes using Treedit (Rambault & Charleston, 2001).

As our phylogenetic reconstruction was based on only half of the species of the genus, we also checked the association between characters described above, on the whole genus, using simple χ^2 tests; these do not take into account the phylogenetic relationships of the species and therefore erroneously treat each species as independent data points but they give an overview of whether these associations are valid in the whole genus. For each of these tests, characters were treated as binary characters (0: absence, 1: presence for wax and viscoelasticity; 0: narrow, 1: infidibular for pitcher shape, 0: <1000m, 1: >1000m for altitude).

Pitchers of seedlings

One of the aims of this study was to understand the origin of the viscoelastic fluid and the waxy layer and test their possible ontogenic losses in some species. We observed in the field that very young *Nepenthes* individuals (< 10 cm tall), i.e. seedlings, produced a third type of pitcher, later referred as 'seedling' pitcher, that is not a homothetic reduction of a lower pitcher. We described some of these pitchers in the field, in July-August 2009, on separate plants of the species *N. bicalcarata*, *N. ampullaria*, *N. rafflesiana* var. *elongata*, *N. rafflesiana* var. *typica* and *N. gracilis*, growing in sympatry in a mixed peat swamp and heath forest (4°44'N, 114°35'E) of Brunei Darussalam (northern Borneo). The number of internodes between the node bearing the pitcher and the cotyledon scars was estimated. We measured the viscoelasticity of these pitchers and noted whether they bore a waxy layer.

Results

Distribution and association patterns of viscoelasticity, wax and pitcher morphology in the Nepenthes *genus*

53 out of the 74 digestive liquids tested were viscoelastic and five species not sampled here are also reported in the literature to be viscoelastic (McPherson, 2009)78 digestive liquids tested were viscoelastic. This character hence appears to be widespread in the genus (Table 1). Thirty four of these viscoelastic species have a mean relaxation time lower than 0.2 s (min = 0.03 s, max 1.14s, mean = 0.24 s, median = 0.11 s), below this threshold a rapid camera is required to assess the viscoelastic

character of the fluid. The liquids of all *Drosera* and *Drosophyllum* also showed profiles of thinning characteristics of viscoelastic fluids which confirms a preliminary study in *Drosera* (Erni *et al.*, 2008).

82 species out of 107 species for which we managed to gather the information had lower pitchers with a waxy layer. Among these 82 species, 23 did not have any wax on their upper pitchers. Among the 27 species that have lower pitchers lacking wax, none had wax on their upper pitchers.

The Figure 2 provides a global view of the distribution of pitcher forms for both lower and upper pitchers and the morphological and functional transitions observed within the genus. Lower pitcher morphologies are clearly dominated by the ovate-cylindrical form. The infundibular and ovate/cylindrical forms are the most often observed forms among upper pitchers. Ellipsoidal forms found in lower pitchers are never found among upper pitchers. The majority of species do not exhibit a marked morphological transition as pointed out by the diagonal of the matrix.

Chi-square tests revealed that a narrow pitcher shape is significantly associated with the presence of waxy zone in upper pitchers while the infundibular shape is almost always associated with the absence of wax (Table 2).

Table 2 indicates that apart from this association, the viscoelasticity-altitude association is marginally significant: viscoelastic species tend to be more often found in mountainous environments as already noticed in a previous study (Bonhomme *et al.*, in prep.-c). Similarly, the morphology-altitude association is also marginally significant: infundibular pitcher shapes are often associated with a mountainous environment.

Phylogenetic reconstruction of ancestral character states and test of correlated evolution

The Figures 3 illustrates the reconstruction of ancestral states for the presence of a waxy layer on lower pitchers and upper pitchers and the Figure 4 the reconsctruction of ancestral state for the presence of a viscoelastic fluid inside the pitcher. The presence of a waxy layer in lower pitchers is unambiguously plesiomorphic, and MP reconstructions inferred 12 transitions for this character (Figure 3). Our reconstruction suggests that the viscoelastic character is probably also plesiomorphic and has been lost several times independently (9 steps estimated by MP). Concerning the presence of a waxy layer in upper pitcher, ancestral character state at the root is ambiguous, however our reconstruction shows that there has been many evolutionary transitions for this character: wax has been lost and/ or acquired in upper pitchers 11 times during the evolution of the genus. Concerning pitcher shapes, our reconstructions suggest that the ancestral shape for lower and upper pitcher is the narrowed shape and that the changes in shape during the transitions from lower to upper pitchers have occurred repeatedly during evolution (15 steps estimated by MP – Figure 3). It must be noticed that this phylogeny only gathers 50 out of the 120 species described and that this non exhaustive sampling and the fact that the phylogeny is poorly resolved may influence our conclusions. This phylogeny nevertheless illustrates a global lack of phylogenetic clustering of *Nepenthes* species exhibiting similar pitcher characteristics, and thus a relative evolutionary lability of the traits investigated.

We then performed tests of correlated evolution using the phylogeny (Table 2). The same highly significant correlation between the presence of a waxy layer and the pitcher morphology appeared in lower and upper pitchers. The association between the viscoelasticity of the liquid and the presence of a waxy layer in upper pitchers is marginally significant: the species with no waxy layer on their upper pitchers always have a viscoelastic fluid, but there are a lot of species that have both a waxy layer on their upper pitchers and a viscoelastic fluid which makes the correlation only marginally significant (Table 2, Figure 4). The other striking association is the association between losses of wax during the transition from lower to upper pitchers and changes in pitcher morphology: species that have “narrow” lower pitchers and “infundibulate” upper pitchers almost systematically lose their waxy layer on their upper pitcher, and interestingly they also all have a viscoelastic digestive liquid. Other associations between upper pitcher morphology, VE, waxy layer on upper pitchers and altitudinal range of species were not significant according to this test.

Identification of a third ontogenic type of pitcher

Observations on the miniature pitchers of seedlings brought two important results. First, the relaxation times measured on the fluid of seedling pitchers revealed that even the species (*N. ampullaria*, *N. bicalcarata*, *N. gracilis*) that were thought to never have a viscoelastic fluid, do have a viscoelastic fluid in their early development (Table 2, Figure 4). Moreover the fluid relaxation times measured on these seedling pitchers seem to be longer than those measured on the juvenile pitchers produced later in the plant ontogeny (Table 1, 2). Similarly, a waxy layer is observed in all the species investigated, even in species previously thought to never bear a waxy layer (Figure 5). Rough age estimations are given for information and should be considered only as indicative data: our aim was simply to investigate whether the juvenile characters in these species are shared between species and are different from the pitchers produced later in the plant’s ontogeny.

Discussion

Viscoelasticity is a plesiomorphic character shared with Drosera *and* Drosophyllum

The survey of 75 out of 120 species indicates that the viscoelasticity of the digestive liquid is a widespread character within the genus. Viscoelasticity had already been hypothesized to be more widespread than previously thought and that *Nepenthes* should no longer be considered as simple gravity-based pitfall traps (Gaume & Forterre, 2007), and our data confirm this view.

As suggested by the reconstruction of ancestral character states, viscoelasticity could be a plesiomorphic character in the genus *Nepenthes*. However, a better resolved phylogeny encompassing more species is necessary to confirm this suggestion. Similar behavior of the fluid of the closely related genera of *Nepenthes*, *Drosera* and *Drosophyllum*, suggests that it could be a shared character inherited from their common ancestor and secondarily & independently lost several times. This view has also been previously hypothesized (Gaume & Forterre, 2007, McPherson, 2009) but is here supported with a phylogeny and observations on sister genera. The viscoelastic properties used in the sticky traps produced by the related lineages, *Drosera* and *Drosophyllum* have probably conferred an immediate advantage for the leaves modified as pitchers of the *Nepenthes* ancestor.

The third type of pitcher evidenced here, i.e. the seedling one, produced in the first ontogenic stages has a viscoelastic digestive liquid pitchers. We hypothesize that all species of the genus, if investigated very early in their ontogeny, will exhibit such a viscoelastic behavior on their digestive liquid.

Plesiomorphic origin of the waxy layer and its multiple losses

The reconstruction of ancestral character states suggests that the presence of a waxy layer in the lower pitchers is also a plesiomorphic character. Yet roughly similar to the wax of other terrestrial plants (Barthlott, 1990, Juniper, 1995, Koch *et al.*, 2004, Dragota & Riederer, 2007), the epicuticular waxy layer of *Nepenthes* probably derivates its trapping and structural properties from chemical changes including new constituents and changes in the abundances of shared constituents compared to non-carnivorous plants (Gaume *et al.*, 2002, Riedel *et al.*, 2003, Gorb *et al.*, 2005, Riedel *et al.*, 2007). The use of epicuticular wax as a retentive device in *Nepenthes* is not a unique case among carnivorous plants and can even be observed in other pitfall plants, such as the bromeliads *Brocchinia reducta* (Givnish *et al.*, 1984) and *Catopsis berteroniana* (Gaume *et al.* 2004). Investigation of seedling pitchers suggests that, similarly to viscoelastic properties, plesiomorphic characters are expressed very early in plant ontogeny. Further phylogenies will confirm this hypothesis but loss of wax throughout ontogeny is likely a derivate character and has occurred several times. Even if our data indicate an ambiguous character state at the root of the tree, the presence of wax on the “western” species found to be the most basal in all phylogenies published so far (Meimberg & Heubl, 2006, Bonhomme *et al.*, in prep.-c) supports this hypothesis.

The link between pitcher form and function

All the pitcher shapes observed in lower pitchers are never observed in upper pitchers and many of the possible morphological transitions between pitcher shapes are never observed. This suggests that pitcher shape is under strong mechanical and/or functional constraints. First, as proposed by Gaume and Di Giusto (2009) on the *Nepenthes rafflesiana* complex and confirmed here on the whole genus, the presence of a waxy zone necessarily implies an elongate-narrowed pitcher morphology (Gaume & Di Giusto, 2009b). By contrast, the absence of the waxy zone allows a larger diversity of pitcher shapes, particularly for upper pitchers that present different degrees and forms of ‘infundibularity’. Our results clearly demonstrate that the loss of wax during ontogeny is accompanied by a change in pitcher morphology. We can reasonably hypothesize that the most efficient position for a pitcher to trap insects is the vertical position, *a fortiori* when it is covered with a slippery waxy layer. But when this layer is absent, the pitcher then relies on its fluid to trap insect and the constraint on the pitcher shape is thus probably alleviated.

As suggested by the transition matrix, all possible transitions are not observed. The tendril that bears the upper pitchers is systematically circumvoluted and this may not be neutral as regards the morphology of these pitchers. Fine characterization of pitcher morphology, e.g. geometrical morphometrics on the pitcher shape, would be of first interest in testing the impact of the tendril production on the shape of upper pitchers.

The pitcher shapes observed in the genus are probably explained by a mixture of mechanical constraints and adaptive causes. What are then the factors of selection that can explain part of the diversity observed?

Causes of the functional and morphological diversity of pitchers in the genus Nepenthes

The association found between studied traits do not strongly support previous results (Bonhomme *et al.*, in prep.-c) since they show that viscoelasticity and wax are not significantly negatively associated in both the test conducted on all species and the analysis using the phylogenetic reconstruction. We must however temper these results. First, we have used binary characters but Bonhomme et al. (submitted) showed a tradeoff between quantitative production of wax and viscoelasticity (Bonhomme *et al.*, submitted to *Functional Ecology*). Then, tests that take into account quantitative measurement of wax and VE as well as a more exhaustive and better resolved phylogeny and an exhaustive census on the viscoelastic properties of the digestive fluids, could lead to another view of the correlated evolution of the traits analyzed here.

We did not either observe an association pattern between non-viscoelastic fluids and lowland species (Bonhomme et al. submitted) but this might be because most of the species used for the phylogeny reconstruction were montane species. Hence our sampling is for now very biased and again a more exhaustive and better resolved phylogeny could lead to different conclusions. The entomofauna present in the environment the plant colonizes should represent an important pressure on the evolution of its trapping strategy(Bonhomme *et al.*, submitted to *Functional Ecology*). Ecological studies that define ecological conditions in a more detailed way (vegetation, potential prey spectra) need to be carried out to explore this question in depth. We must also keep in mind that the relationships of traits associated with the carnivorous syndrome are probably complex and the carnivorous syndrome also involves characters, such as the peristome shape, that are not taken into account here.

An evo-devo model for the evolution of the trapping strategies

“We are all born crazy. Some remain that way.” – Samuel Beckett, *Waiting for Godot*.

This also applies to the *Nepenthes* pitchers if “crazy is replaced with “waxy” “or viscoelastic”. Our data suggest that plesiomorphic characters (presence of a waxy layer and a viscoelastic fluid) are expressed in seedling pitchers and then lost or retained throughout ontogeny. A very simple model illustrates this view and unifies all the dynamics of wax and viscoelasticity losses observed so far (Figure 6). For instance wax and viscoelasticity are lost very early for *N. ampullaria* and *N. bicalcarata* (trajectory A – Figure 6). By contrast, *Nepenthes rafflesiana* var. *typica* loses its waxy layer during the transition from self-supporting-stage to climbing stage (trajectory B – Figure 6) whereas *N. rafflesiana* var. *elongata* always presents a waxy layer (trajectory C – Figure 6) (Gaume & Di Giusto, 2009a). Trajectories C’ and C’’ illustrate hypothetical ontogeny patterns where wax/viscoelasticity could be partially reduced or totally lost, but later in the plant life. In this model, all the seedling pitchers are both waxy and viscoelastic and the trapping strategy they exhibit at the adult age simply corresponds to more or less rapid ontogenic losses or retention of the waxy layer and/or the viscoelasticity of their liquid ($\theta_{A,B,C}$ – Figure 6). Our census indicates that wax is never secondary acquired during ontogeny but has been instead lost in at least 23 species (Table 1). These timings and onsets of the expression of these retentive devices suggest that heterochrony (Gould, 1977) is a possible mechanism for the evolution of new pitcher forms and associated trapping strategy in the *Nepenthes* genus and could easily explain part of the morphological diversity observed in this species-rich carnivorous genus (Gaume & Di Giusto, 2009b). The main concept of such evo-devo models is that little developmental changes can lead to major functional shifts, here illustrated for example as the deletion of the waxy layer in the pitcher developmental pattern which leads to new pitcher form and function (Gaume & Di Giusto, 2009b). An exhaustive census of seedling pitchers, the recording of the fluid behavior for the upper pitchers of all *Nepenthes* species and the remaining lower pitchers would allow to test the hypothesis that, similarly to the waxy layer, viscoelasticity is never acquired but can be lost more or less early throughout ontogeny.

Studies on the dynamics of the developmental loss of the waxy layer will refine the simplistic evo-devo model presented here. ‘Node by node’ monitoring of a larger number of species throughout their ontogeny will permit to test the validity of this model.

Species	Altitude	Lower pitcher			Upper pitcher		Species	Altitude	Lower pitcher			Upper pitcher	
		Waxy layer	Shape	RT (s)	Waxy layer	Shape			Waxy layer	Shape	RT (s)	Waxy layer	Shape
N. adnata	montane	yes	cyl	-	yes	cyl	*N. macrophylla*	montane	yes	cyl	0.04	yes	cyl
N. alata	montane	yes	ov-cyl	0.04	yes	ov-cyl	*N. macrovulgaris*	lowland	yes	cyl	NonVE	yes	cyl
N. alba	montane	yes	glob-cyl	-	yes	glob-cyl	*N. madagascariensis*	lowland	yes	ov-cyl	NonVE	yes	ov-cyl
N. albomarginata	lowland	yes	cyl	0.03	yes	cyl	*N. masoalensis*	lowland	yes	ov-cyl	-	yes	ov-cyl
N. ampullaria	lowland	no	ov	NonVE	no	infun	*N. maxima*	montane	yes	cyl	1.04	no	infun
N. angasanensis	montane	yes	ov-cyl	-	yes	ov-cyl	*N. merrilliana*	lowland	no	ellip	NonVE	no	infun
N. argentii	montane	no	infun	0.20			*N. micramphora*	montane	yes	glob-cyl	-	yes	glob-cyl
N. aristolochioides	montane	no	infun-narrow	0.17	no	infun-	*N. mikei*	montane	yes	ov-cyl	NonVE	yes	ov-cyl
N. attenboroughii	montane	no	infun	VE	no	infun	*N. mindanaoensis*	lowland	yes	glob-cyl	0.03	yes	glob-cyl
N. bellii	lowland	no	ellip	0.05	no	infun	*N. mira*	montane	no	ellip	0.16	no	infun
N. benstonei	lowland	yes	glob-cyl	NonVE	yes	glob-cyl	*N. mirabilis*	lowland	yes	ov-cyl	NonVE	yes	cyl
N. bicalcarata	lowland	no	ellip	NonVE	no	infun-cyl	*N. muluensis*	montane	yes	ov-cyl	-	yes	ov-cyl
N. bokoriensis	lowland	yes	ov-cyl	0.08	no	infun-cyl	*N. murudensis*	montane	yes	glob-cyl	-	yes	glob-cyl
N. bongso	montane	yes	ov-cyl	0.13	no	infun	*N. naga*	montane	yes	ov-cyl	-	yes	infun-cyl
N. boschiana	montane	yes	ov-cyl	NonVE	yes	ov-cyl	*N. neoguineensis*	lowland	yes	ov-cyl	0.15	yes	infun-cyl
N. burbidgeae	montane	no	ellip	0.64	no	infun	*N. northiana*	lowland	yes	ellip	NonVE	no	infun
N. burkei	montane	no	ov-cyl	0.03	no	glob-infun	*N. ovata*	montane	no	infun-cyl	0.23	no	infun
N. campanulata	lowland	yes	glob-cyl	0.28	yes	infun	*N. papuana*	lowland	yes	ov-cyl	-	yes	ov-cyl
N. chaniana	montane	yes	ov-cyl	NonVE	no	infun	*N. peltata*	montane	no	ov	-	no	ov
N. clipeata	lowland	yes	glob-cyl	NonVE	yes	glob-cyl	*N. pervillei*	lowland	yes	glob-cyl	0.26	yes	glob-cyl
N. copelandii	montane	yes	cyl	0.46	no	infun	*N. petiolata*	montane	yes	glob-cyl	0.12	yes	glob-cyl
N. danseri	lowland	yes	ov-cyl	1.00	yes	infun-cyl	*N. philippinensis*	lowland	yes	ov-cyl	-	yes	ov-cyl
N. deaniana	montane	no	ellip	-	no	infun	*N. pilosa*	montane	yes	ov-cyl	-	no	infun
N. densiflora	montane	no	ov-cyl	0.07	no	infun	*N. pitopangii*	montane	yes	ov-cyl	VE	no	trump
N. diatas	montane	yes	ov-cyl	NonVE	yes	ov-cyl	*N. platychila*	montane	yes	ov-cyl	-	no	infun
N. distillatoria	lowland	yes	ov-cyl	0.03	yes	ov-cyl	*N. rafflesiana*	lowland	yes	ellip	0.53	no	infun
N. dubia	montane	no	infun-cyl	0.90	no	trump	*N. rajah*	montane	no	ellip	0.14	no	infun
N. edwardsiana	montane	yes	ov-cyl	-	yes	ov-cyl	*N. ramispina*	montane	yes	ov-cyl	NonVE	yes	infun-cyl
N. ephippiata	montane	no	ov-cyl	NonVE	no	glob-infun	*N. reinwardtiana*	montane	yes	ov-cyl	0.05	yes	ov-cyl
N. eustachya	montane	yes	ov-cyl	NonVE	yes	ov-cyl	*N. rhombicaulis*	montane	yes	ov-cyl	-	-	-

Species	**Altitude**	**Lower pitcher**			**Upper pitcher**	
		Waxy layer	**Shape**	**RT (s)**	**Waxy layer**	**Shape**
N. eymae	montane	yes	ov-cyl	0.10	no	trump
N. faizaliana	montane	yes	ov-cyl	NonVE	yes	ov-cyl
N. flava	montane	no	infun	VE	no	trump
N. fusca	montane	yes	cyl	1.14	no	infun
N. glabrata	montane	yes	ov-cyl	0.09	yes	infun-cyl
N. glandulifera	montane	yes	ov-cyl	-	no	infun
N. gracilis	lowland	yes	ov-cyl	NonVE	yes	ov-cyl
N. gracillima	montane	yes	ov-cyl	0.04	yes	ov-cyl
N. gymnamphora	montane	yes	ov-cyl	0.04	yes	cyl
N. hamata	montane	yes	ov-cyl	0.03	yes	ov-cyl
N. hispida	lowland	yes	glob-cyl	-	yes	glob-cyl
N. hurreliana	montane	no	infun	-	no	infun
N. inermis	montane	yes	ov-cyl	0.27	no	trump
N. insignis	lowland	yes	ov-cyl	0.06	no	infun-cyl
N. izumiae	montane	yes	ov-cyl	-	yes	ov-cyl
N. jacquelinae	montane	no	infun	VE	no	infun
N. jamban	montane	no	infun	VE	no	trump
N. kampotiana	lowland	yes	glob-cyl	-	yes	glob-cyl
N. khasiana	montane	yes	glob-cyl	0.05	yes	glob-cyl
N. kongkandana	lowland	yes	ov-cyl	-	yes	cyl
N. lamii	montane	yes	ov-cyl	0.11	yes	infun-cyl
N. lavicola	montane	yes	ov	-	yes	ov
N. lingulata	montane	yes	cyl	-	yes	cyl
N. longifolia	lowland	yes	ov-cyl	0.41	yes	ov-cyl
N. lowii	montane	yes	ov-cyl	NonVE	no	glob-infun
N. macfarlanei	montane	no	infun	0.09	no	infun
N. rigidifolia	montane	yes	ov-cyl	-	yes	infun-cyl
N. rowanae	lowland	yes	ov-cyl	-	no	infun-cyl
N. sanguinea	montane	yes	ov-cyl	0.08	yes	infun-cyl
N. saranganiensis	montane	yes	ov-cyl	-	yes	ov-cyl
N. sibuyanensis	montane	no	ellip	0.23	no	infun-cyl
N. singalana	montane	yes	ov-cyl	0.04	yes	ov-cyl
N. smilesii	lowland	yes	ov-cyl	-	yes	cyl
N. spathulata	montane	yes	ov-cyl	0.34	yes	ov-cyl
N. spectabilis	montane	yes	ov-cyl	0.41	yes	infun-cyl
N. stenophylla	montane	yes	ov-cyl	0.03	yes	infun-cyl
N. sumatrana	lowland	yes	ellip	-	no	infun
N. surigaonensis	montane	no	ellip	-	no	infun
N. talagensis	montane	no	infun	0.45	no	infun
N. tenax	lowland	yes	ov-cyl	-	yes	infun-cyl
N. tentaculata	montane	yes	glob-cyl	0.06	yes	glob-cyl
N. tenuis	montane	no	infun	0.75	no	trump
N. thorelii	lowland	yes	ellip		no	infun
N. tobaica	montane	yes	ov-cyl	0.24	yes	ov-cyl
N. tomoriana	lowland	yes	ov-cyl	0.03	yes	infun-cyl
N. treubiana	lowland	yes	ov-cyl	-	no	infun-cyl
N. truncata	lowland	yes	ov-cyl	NonVE	no	cyl
N. veitchii	lowland	yes	ellip	0.03	no	infun
N. ventricosa	montane	no	glob-infun	NonVE	no	glob-infun
N. vieillardii	lowland	yes	ov-cyl	0.07	-	-
N. villosa	montane	yes	ov	0.15	yes	infun-cyl
N. vogelli	montane	yes	infun	0.05	no	infun

Tables

Table 1 (previous page): Species and characters used in this study. Relaxation time (RT) are proportional to viscoelasticity, those indicated VE are reported by McPherson to have a viscous fluid (McPherson, 2009).

Character associations	**χ^2 all species (1 df)**					**Pagel's test on ITS phylogeny**		
	Sense of association	**N**	**χ^2**	**df**	***P* =**	**N**	**Δ log(likelihood)**	***P* =**
VE – wax UP	VE+/non-waxy	76	0.14	1	0.71	43	1.53	**0.09**
VE - shape UP	VE+/infundibular	74	0.41	1	0.52	36	1.56	0.19
Wax LP- shape LP	Waxy+/narrow	105	30.28	1	< **0.001**	45	6.17	**<0.01**
Wax UP - shape UP	Waxy+/narrow	89	73.39	1	< **0.001**	43	25.1	< **0.01**
Loss of wax- change in pitcher shape	non-waxy/ infundibular	86	34.20	1	< **0.001**	43	9.44	< **0.01**
Shape UP - Altitude	narrow/lowland	88	3.07	1	**0.08**	50	1.29	> 0.5
VE - Altitude	VE+/montane	76	3.85	1	**0.05**	43	0.81	> 0.5
Wax UP- Altitude	non-waxy/ montane	107	0.91	1	0.34	50	1.4	> 0.5

Table 2: Dependence tests calculated on all species (χ^2) and using the ITS phylogeny (Pagel's test). UP stands for upper pitchers, LP stands for lower pitchers.

Species	**Estimated age (internodes)**	**Relaxation time (s)**
N. ampullaria	10	2.80
N. ampullaria	15	0.50
N. ampullaria	30	0.10
N. ampullaria	35	0.00
N. bicalcarata	10	0.34
N. bicalcarata	15	0.00
N. bicalcarata	20	0.00
N. gracilis	15	0.10
N. gracilis	25	0.10
N. gracilis	35	0.10
N. rafflesiana var. *elongata*	20	1.40
N. rafflesiana var. *elongata*	25	1.80
N. rafflesiana var. *elongata*	30	0.40
N. rafflesiana var. *elongata*	30	0.50
N. rafflesiana var. *elongata*	8	1.00
N. rafflesiana var. *elongata*	10	4.00
N. rafflesiana var. *elongata*	15	4.40
N. rafflesiana var. *typica*	20	10.70
N. rafflesiana var. *typica*	25	0.90
N. rafflesiana var. *typica*	30	12.00

Table 3: List of the seedling pitchers whose relaxation time has been measured.

Figures

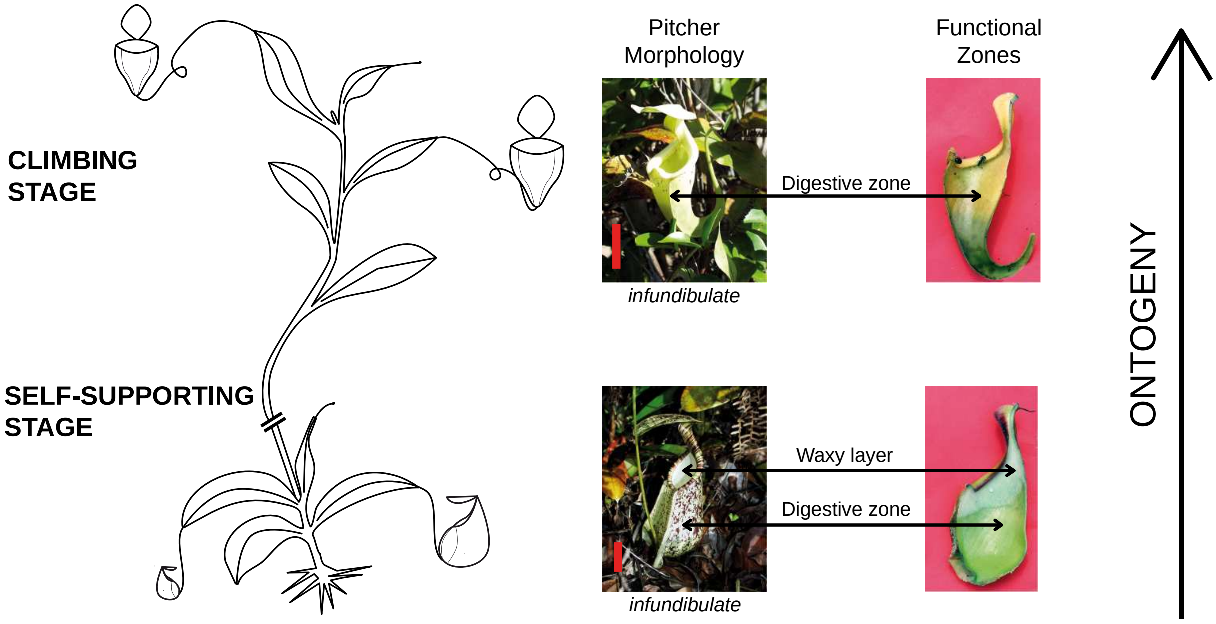


Figure 1: Example of pitcher dimorphism in *Nepenthes rafflesiana* var. *typica*. Most of *Nepenthes* plants are vines characterized by a pitcher-dimorphism, with young rosettes or self-supporting stages exhibiting terrestrial pitchers of the ‘lower’ type, and older climbing stages exhibiting aerial pitchers of the ‘upper’ type. In *Nepenthes rafflesiana*, the ‘lower’ pitchers are ellipsoidal and/or elongate while the ‘upper’ pitchers are infundibular. This pitcher shape polymorphism is associated with a polymorphism concerning the functional zones. In *Nepenthes rafflesiana* var. *typica*, ‘lower’ pitchers of young plants bear a slippery waxy zone. This character is lost throughout ontogeny and ‘upper’ pitchers never bear a waxy zone. Red bars correspond to 5 cm.

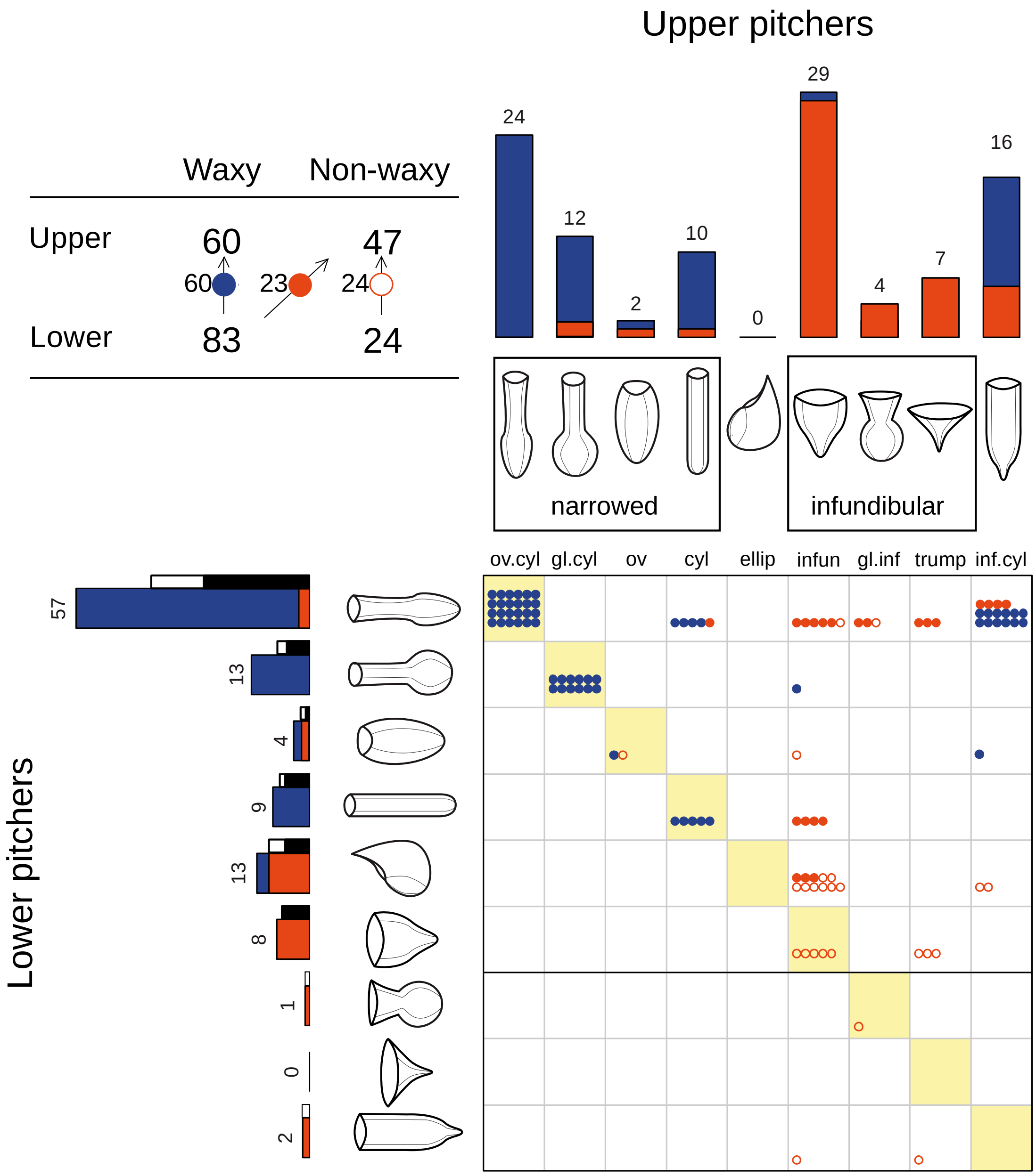
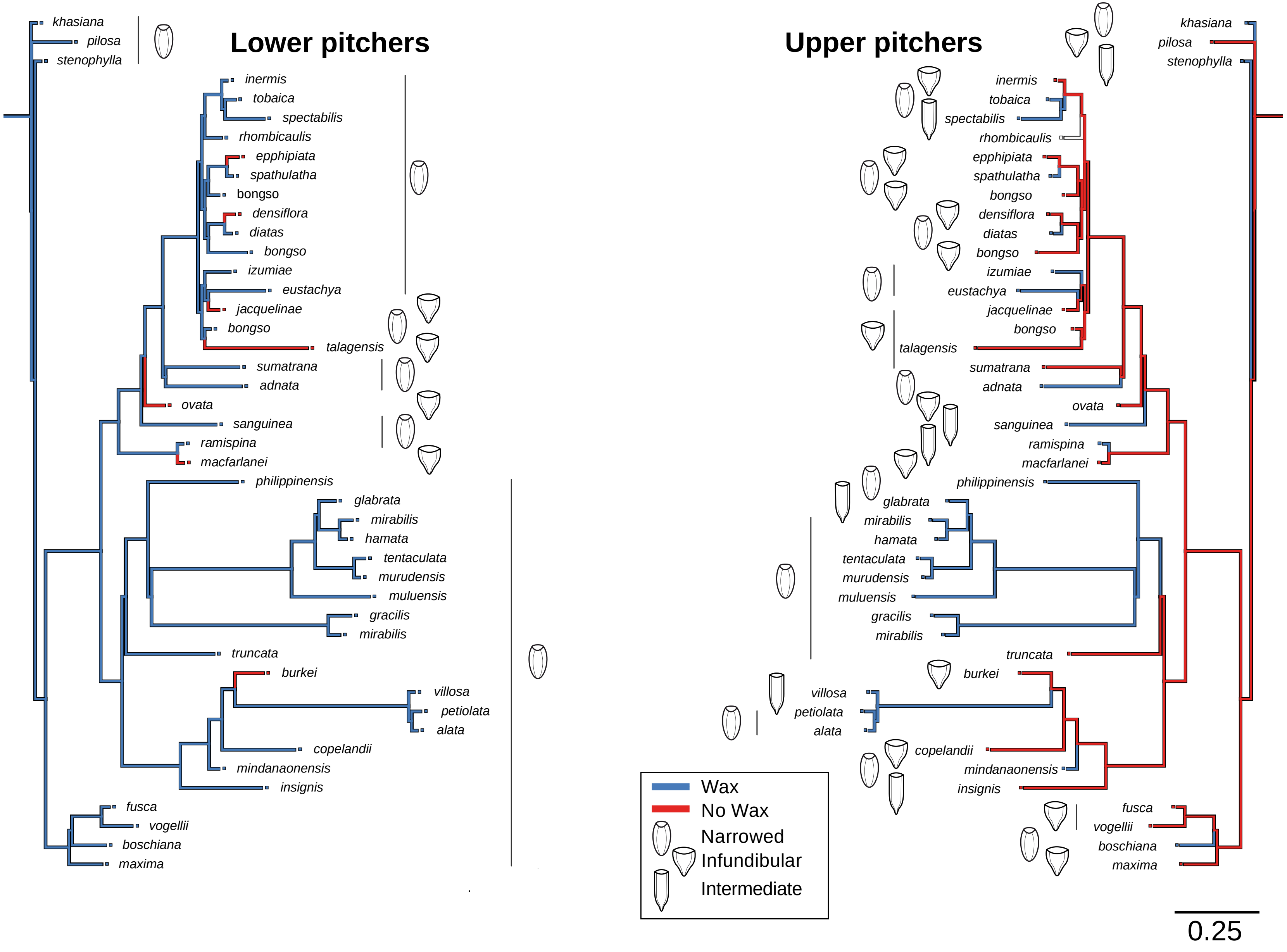


Figure 2: Transition matrix for pitcher morphologies and associated retentive devices. Dot represents single species transition from a peculiar lower pitcher morphology (left) to an upper pitcher morphology (top). The numbers of species that did not change in pitcher morphology throughout ontogeny are found in the diagonal of the matrix. Filled orange dots correspond to species that lost their waxy layer throughout ontogeny; empty orange dots correspond to species that do bear a waxy layer neither in lower nor in upper pitchers. Filled blue dots correspond to species whose both lower and upper pitchers possess a waxy layer. The number of transitions is resumed in the table and their frequency for each pitcher shapes are indicated in the histograms. The thinner bars correspond to the number of species reported to possess a viscoelastic digestive liquid (black) on their lower pitchers out of the number of species available for each pitcher shape (whole rectangle). The waxy layer could be lost but is never acquired throughout ontogeny.



Lower pitchers

Upper pitchers

Figure 3 (previous page): Reconstruction of ancestral character states for presence of a waxy layer in lower (left) and in upper (right) pitcher and for the associated pitcher shape. Reconstruction of the ancestral states is drawn using Maximum Parsimony optimization criterion. Pitcher shapes have been reduced to three categories: narrowed, infundibular and intermediate, the latter have been excluded from the correlation analysis. Almost all lower pitchers present a waxy layer that has been lost several times in upper pitchers.

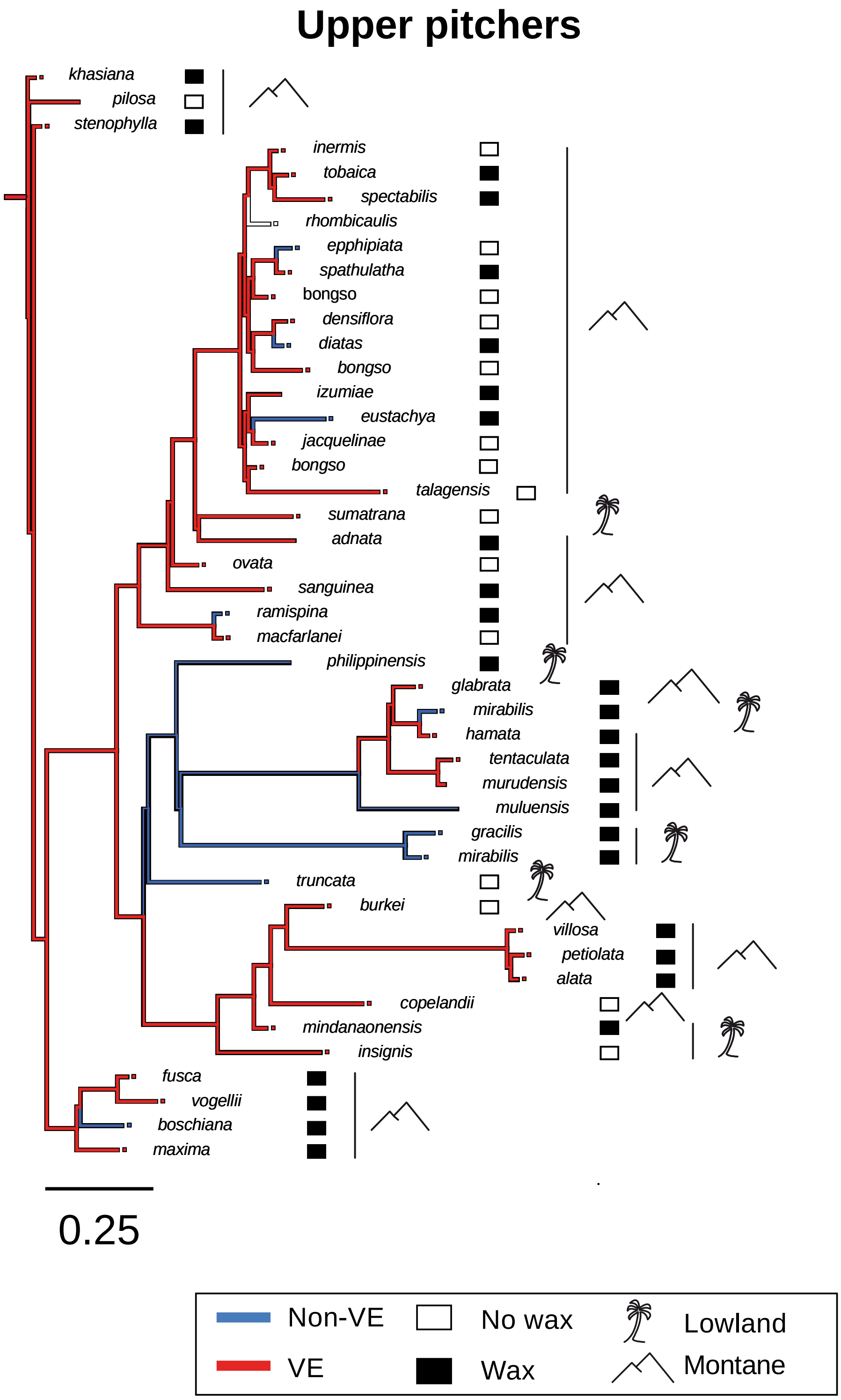


Figure 4: Reconstruction of ancestral character states for presence of a viscoelastic fluid and for the associated presence of a waxy layer and ecology. Reconstruction of the ancestral states is drawn using Maximum Parsimony optimization criterion. Pitcher shapes have been reduced to three categories: narrowed, infundibular and intermediate, the latter have been excluded from the correlation analysis. The presence of a viscoelastic fluid is a plesiomorphic character lost several times. There is tendancy for the species that are not viscoelastic to bear a waxy layer. Altitude is not associated with the presence of a viscoelastic fluid or a waxy layer in upper pitcher on this phylogeny.

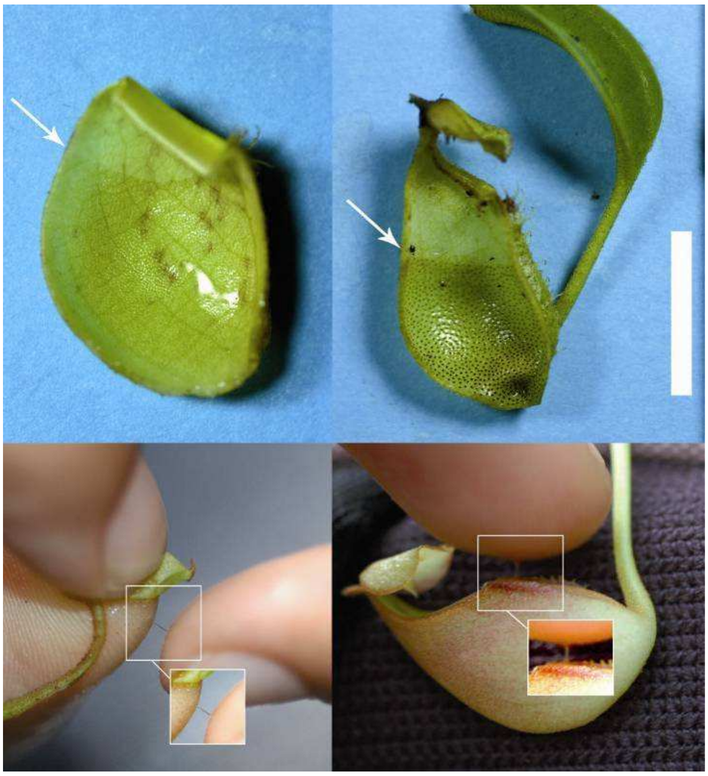


Figure 5: *Nepenthes ampullaria* (left) and *N. bicalcarata* (right) present waxy walls and viscoelastic fluid in their 'seedling' pitchers. These species, which never bear a waxy layer (up) and do not have a viscoelastic fluid (down and Table 2) at the 'lower pitcher' juvenile stage are here reported to possess these two characters on their pitchers at the seedling stage, but which are lost quite early in plant ontogeny. Arrows indicate the frontier between the digestive and the conductive zones. The white bar corresponds to 5 mm for all pictures.

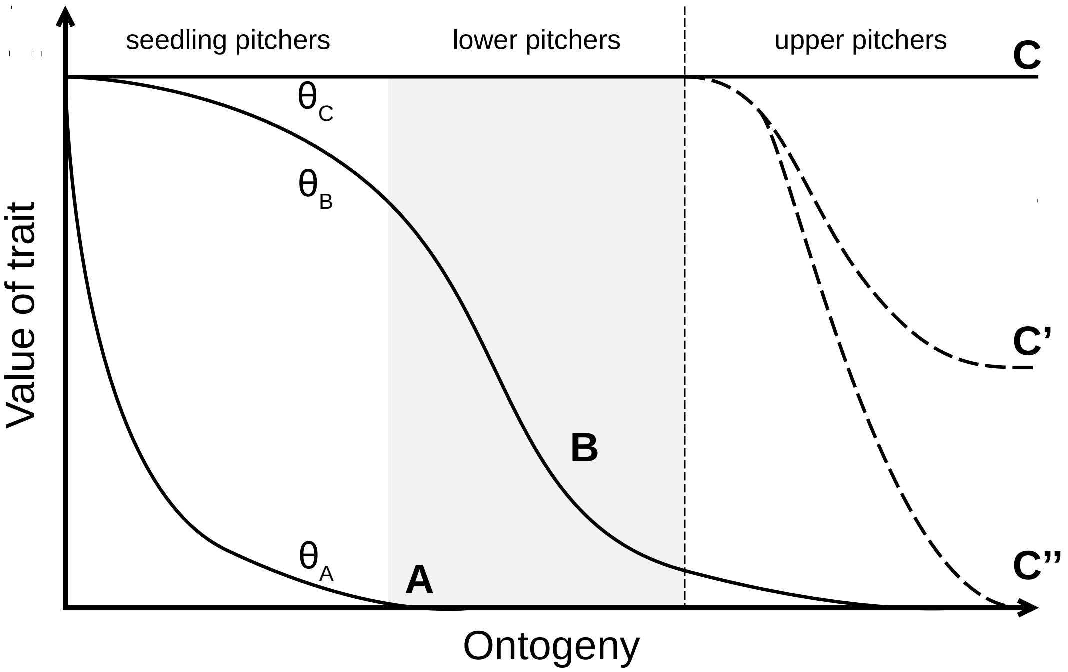


Figure 6: A developmental model that gathers the different patterns of loss or retention of carnivorous traits throughout ontogeny. The y-axis refers to the presence of a viscoelastic fluid or the proportion of the waxy layer to the total pitcher length; the x-axis refers to the plant ontogeny (e.g. the number of internodes since germination). Three ontogenic stages are represented including the 'seedling' stage evidenced here. The different trajectories represent patterns described so far: A corresponds to the lost of the waxy layer and the viscoelastic fluid for *N. ampullaria* and *N. bicalcarata*, B corresponds to the loss of wax in *N. rafflesiana* var. *elongata* versus A that represents its retention in the variety *typica* (Gaume & Di Giusto, 2009).

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Bonhomme, V., Pelloux-Prayer, H., Jousselin, E., Forterre, Y., Labat, J.-J. & Gaume, L. (submitted to *Functional Ecology*) Slippery or sticky? Functional diversity in the trapping strategy of *Nepenthes* carnivorous plants.

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CONCLUSION

Cette thèse fait le lien entre deux approches, d'une part l'étude des mécanismes de piégeage et de développement de quelques espèces et d'autre part la phylogénie moléculaire du genre, nous a permis d'ébaucher l'histoire évolutive du genre *Nepenthes* et de la diversification de ses mécanismes de piégeage. Nous allons résumer ses principaux résultats, puis faire un tour d'horizon des limites et des perspectives qui émergent de ces travaux.

Diversités fonctionnelle et développementale du genre *Nepenthes*

Plusieurs stratégies de piégeage existent au sein du genre et la plus efficace est celle qui emploie un fluide viscoélastique

Le **Manuscrit I.1** met en évidence plusieurs résultats majeurs pour le genre, représenté par un sous-jeu sélectionné pour couvrir l'ensemble de l'aire de répartition, des modes de vie et des formes de piège. Nous avons montré dans ce genre en miniature la présence d'espèces très cireuses et d'espèces très viscoélastiques. Le caractère viscoélastique n'est donc pas l'apanage de *N. rafflesiana*, chez qui il a été mis en évidence pour la première fois. La quantité de cire produite est également variable : certaines espèces sont très cireuses d'autres très peu. Mieux : parmi les taxa étudiés, les espèces les plus viscoélastiques sont les moins cireuses et vice-versa. Ces résultats suggèrent fortement l'existence pour la plante d'un *trade-off*, l'investissement dans une zone cireuse épaisse se faisant au détriment d'un liquide très viscoélastique. La variabilité des mécanismes de rétention employés ne prend du relief que si elle est mise en perspective avec son influence sur le piégeage. Nous avons donc cherché à comprendre quels sont les effets respectifs de la cire et de la viscoélasticité du liquide sur les insectes que capturent les *Nepenthes* dans leur milieu.

L'efficacité du liquide viscoélastique et celle de la zone cireuse diffèrent selon les insectes

Dans le même **Manuscrit I.1** les taux de rétention des deux principaux types d'insectes capturés sur le terrain, des fourmis et des mouches, ont été mis en lien avec la description précédemment évoquée des stratégies de piégeages. Nous montrons que l'efficacité rétentive de la zone cireuse et du liquide viscoélastique dépend du type d'insecte piégé : le liquide viscoélastique retient très efficacement mouches et fourmis alors que la cire est plus efficace sur les fourmis que sur les mouches. Ces résultats sont les premiers qui mettent en évidence sur plusieurs espèces le lien entre caractéristique du piègeet spectre de proies Ces résultats, mettent à jour les pressions de sélection exercées par l'entomofaune présente dans l'environnement de la plante sur l'évolution de son mécanisme de piégeage.

Ces résultats expérimentaux sont par ailleurs confirmés par la description des spectres de proies des espèces du Brunei. Les travaux de Christine Alaux (Alaux, 2010), co-encadrée par Laurence Gaume et moi-même, montrent par l'analyse et l'identification des contenus d'urnes de trois espèces de *Nepenthes* vivant en sympatrie dans les forêts tourbeuses du Brunei que *N. gracilis*, aux urnes cireuses dépourvues de liquide viscoélastique, capture quasi-exclusivement des insectes rampants. À l'inverse, les urnes terrestres de *N. rafflesiana* var. *typica*, cireuses et au fluide élastique, capturent une plus grande diversité d'insectes. Les urnes aériennes non-cireuses et au fluide très élastique de la même espèce, capturent la plus grande diversité d'insectes observée à ce jour, et notamment une grande proportion d'insectes volants. La dernière espèce dont les contenus d'urnes ont été décrits est *N. bicalcarata*, qui capture également quasi-exclusivement des insectes rampants, qui n'est ni cireuse, ni viscoélastique. Elle est revanche en association avec une fourmi et nos travaux ont cherché à clarifier la nature de l'interaction entre la plante carnivore et sa fourmi symbiotique. L'étude comparative des spectres de proies de différentes espèces de *Nepenthes* semble donc être une perspective importante de nos travaux qui permettrait de confirmer nos hypothèses concernant le rôle de l'entomofaune sur l'évolution des caractéristiques des urnes. Ces études cependant sont fastidieuses car elles nécessitent l'identification de proies parfois à moitié digérées. Des méthodes d'identification moléculaires à partir des débris pourraient représenter une application des avancées des programmes de *barcoding* mais ces méthodes nécessiteraient néanmoins des mises au point techniques afin d'amplifier de l'ADN probablement dégradé.

Une stratégie de piégeage alternative : l'association avec une fourmi mutualiste

Nous avons observé sur le terrain que les fourmis mutualistes de *Nepenthes bicalcarata* attaquaient les insectes tombés dans l'urne puis nous avons montré dans le **Manuscrit I.2** {Bonhomme, in press. #1276} que la stratégie de piégeage de *N. bicalcarata* repose sur la présence et l'activité de chasse de cette fourmi. Nous avons mis en évidence l'existence d'un mutualisme de nutrition entre la plante et sa fourmi et montré que l'hébergement d'une fourmi mutualiste constitue une nouvelle stratégie de piégeage pour le genre *Nepenthes*. Nos résultats n'excluent pas la possibilité que la fourmi offre d'autres types de bénéfices à la plante, mis en évidence par d'autres études, à savoir la protection des pièges actifs contre la putréfaction et la protection des pièges en développement contre les attaques d'un charançon phytophage spécifique. Ils clarifient même le paradoxe d'une fourmi mutualiste chez une plante carnivore. *Camponotus schmitzi* n'est agressive envers les visiteurs de sa plante-hôte que lorsqu'ils sont tombés dans l'urne, où elle passe le plus clair de son temps cachée en embuscade sous le péristome, permettant ainsi la visite de nombreux insectes attirés par le nectar extrafloral et constituant des proies potentielles. Par son comportement de chasse en embuscade, la fourmi augmente le taux de capture de la plante. Donc en plus du gîte dont dispose *C. schmitzi* dans les vrilles creuses et les urnes, et du nectar sucré qu'elle consomme, elle bénéficie en permanence d'un stock de protéines à portée de mandibules. La fragmentation des proies de *Nepenthes bicalcarata* et la présence vraisemblable de faeces de *C. schmitzi* dans les urnes suggèrent que ces fourmis mutualistes participent au processus de digestion des proies et se substitueraient aux enzymes de type Nepenthesin dont l'absence est suggérée par la faible acidité du liquide chez cette espèce. Les premiers résultats de l'analyse comparative qui tentera d'établir le lien entre les stratégies de piégeage, l'attraction des visiteurs et la physiologie des urnes des espèces du Brunei montre en effet que les changements de stratégies de piégeages peuvent s'accompagner de changements dans la physiologie des pièges (**Annexe 4**).

Comme discuté dans le Manuscrit II, la plupart des traits atypiques de *N. bicalcarata* (longévité des urnes, tendrils creux, absence de surface cireuse et de liquide viscoélastique, etc.) semblent tous être bénéfiques à *C. schmitzi*. Par ailleurs certains traits des fourmis (nage, chasse en embuscade, etc.) semblent eux aussi particulièrement « adaptés » à leur association avec leurs plantes hôte. Ceci suggère qu'il peut exister une coévolution entre les deux partenaires qui inclue même des adaptations physiologiques des urnes et des fourmis.

Contraintes développementales sur la production d'urnes

Un des objectifs de cette thèse était de fournir la première description architecturale[4] du genre *Nepenthes*. Nous avons finalement utilisé les concepts de l'architecture végétale pour étudier les contraintes développementales sur la production d'urnes dans deux contextes indépendants.

Premièrement, nous avons les données nécessaires à la description des modalités de croissance et les activités des compartiments carnivore et photosynthétique au cours de l'ontogénie des espèces du Brunei. Le cadre de cette étude déborde de celui de cette thèse mais pour les données présentées dans le Chapitre I en lien avec notre problématique, retenons que les espèces du genre présentent des modalités de croissance différentes et que l'acquisition de la lianescence est reliée à une diminution du nombre d'urnes fonctionnelles produites, au bénéfice probable d'un investissement dans la

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[4] L'architecture végétale étant également *le* domaine de spécialité du laboratoire AMAP, historiquement fondateur de la discipline et actuellement leader mondial dans le domaine.

croissance de tiges vrillées impliquées dans l'accrochage de la liane sur des supports végétaux environnants (Annexe 2).

Deuxièmement, puisque les espèces de *Nepenthes* sont dioïques et entomogames, la nécessité de produire des urnes lourdes et de les maintenir suspendues n'est pas le seul « challenge » développemental. En période de floraison, les insectes sont utilisés par les plantes carnivores comme proies mais aussi comme pollinisateurs. On s'attend donc comme discuté dans le **Manuscrit I.3** à ce que des mécanismes minimisent ce conflit évolutif appelé conflit pollinisateur-proie. Nous montrons pour la première fois chez *Nepenthes*, qu'un mécanisme développemental baptisé *stop-and-go* existe et permet de minimiser le conflit pollinisateur-proie par un double décalage spatial et temporel de l'activité des urnes et des fleurs. Spatial, car lors de la floraison, les urnes fonctionnelles sont situées plus loin de l'apex où la floraison a lieu, et que ce n'est pas le cas en dehors des périodes de floraison. Temporel, car le développement des urnes proche de l'apex est en fait arrêté au moment de la floraison pour être repris de façon synchrone au moment de la fructification. Ce mécanisme original permet donc de minimiser le conflit pollinisateur-proie sans pour autant diminuer l'activité globale des organes carnivores puisque les urnes sont simplement mises en pause durant la floraison. La production de pièges actifs n'est donc pas graduellement échelonnée dans le temps comme pendant la période végétative mais momentanément restreinte pour être simultanément réactivée après la floraison. L'arrivée massive de nutriments qui en résulte coïncide en effet avec la fructification et la ramification sympodiale. Une perspective serait de tester le rôle de l'inflorescence dans l'inhibition des bourgeons apicaux. Si l'inhibition du développement des urnes est causée par les inflorescences, alors des expériences de castration de la plante devraient pouvoir mettre en évidence une levée d'inhibition. Après cette parenthèse développementale, nous allons décrire le deuxième temps majeur de cette thèse, l'obtention d'une phylogénie moléculaire et la reconstruction de l'histoire évolutive du genre.

Histoire évolutive du genre et mécanismes à l'origine de sa diversité

Vers une phylogénie moléculaire résolue

Les premières phylogénies moléculaires du genre datent du début du siècle mais aucune n'était vraiment résolue à ce jour. Un des objectifs de cette thèse était d'obtenir une phylogénie moléculaire suffisamment résolutive pour, au-delà de son intérêt intrinsèque, retracer l'histoire des caractères qui ont conduit à la diversité fonctionnelle mise en évidence dans le Chapitre I.

Nous confirmons que l'obtention d'une phylogénie moléculaire résolue pour le genre tient presque autant de la quête du Saint Graal que d'un travail de paillasse. Le genre *Nepenthes* conjugue les difficultés suivantes : faible taux d'évolution de l'ADN chloroplastique, hybridations fréquentes, niveau de ploïdie incertain, identifications taxinomiques douteuses, statut spécifique attribué sur des critères morphologiques non seulement hétérogènes mais également probablement homoplasiques car impliqués dans les mécanismes de piégeage, dynamique de description de nouvelles espèces quasi exponentielle , et difficulté d'alignement des séquences ADN des groupes externes. Nous présentons malgré tout dans le **Manuscrit II.1** les phylogénies obtenues à partir de 6 empreintes AFLP, 4 marqueurs chloroplastiques (*rps16*, *trnL*, *trnD*, *ycf6*) et l'ITS nucléaire, qui complètent celles actuellement disponibles. Nos données AFLP montrent beaucoup d'homoplasie et ce jeu de données devra probablement être réanalysé après avoir été complété (en ajoutant des empreintes par exemple). L'analyse combinée n'a pas été possible, du fait de l'hétérogénéité des données obtenues dans chacun des six jeux de marqueurs (4 cpDNA, ITS, AFLP) mais les 600 bp de l'ITS sont finalement les plus prometteuses. Des marqueurs supplémentaires sont nécessaires afin d'obtenir une phylogénie résolue. En perspective, les ETS (*external transcribed spacer*), situées à proximité des ITS dans le génome et ayant été utilisées plusieurs fois comme données complémentaires des ITS dans des phylogénies de genres de plantes (e.g. Logacheva et al. 2010) pourraient être testés. Les avancées récentes dans les programmes de barcoding des plantes (Hollingsworth et al.2009 a et b) pourraient également révéler des marqueurs potentiellement informatifs afin de résoudre les phylogénies au niveau intra-genre chez les plantes. Les phylogénies obtenues ne sont pas suffisantes pour décrire le détail de l'histoire biogéographique du groupe dont l'histoire géologique est mouvementée et complexe : composée de changements rapides (les îles des Phillipines, de Bornéo, de Sumatra et la Péninsule Malaise sont périodiquement reliées entre elles par des ponts de terres mais isolés par la ligne de Wallace des îles plus au sud) et de mouvements géologiques plus lents (détachements et déplacements de blocs continentaux). Nous retrouvons certains groupes géographiques sur ces phylogénies mais certaines espèces se trouvent également apparentées à des groupes assez éloignées géographiquement, ce qui suggère des scénarios biogéographiques riches en évènements.

La phylogénie ITS a fourni la phylogénie utilisé dans le **Manuscrit II.2** qui fait d'une part le lien entre l'évolution morphologique et fonctionnelle des espèces du genre et propose d'autre part un mécanisme évo-dévo pour expliquer les diversités actuelles.

Origine et évolution des caractères liés à la carnivorie

Après avoir réalisé à l'échelle du genre l'inventaire exhaustif de la forme et de la présence d'une zone cireuse sur les urnes du bas et des urnes du haut dans la littérature, mesuré le caractère viscoélastique de toutes les espèces présentes dans la collection de Jean-Jacques Labat, résumé l'écologie des espèces avec la répartition altitudinale, nous avons reconstruit l'évolution des traits impliqués dans le piégeage et testé les relations entre chacun de ces traits. Ces reconstructions sont encore incertaines du fait de la faible résolution de la phylogénie mais plusieurs résultats apparaissent de façons assez soutenue.

Le caractère élastique est présent dans la majorité des espèces mesurées (50 sur 71) et présent dans au moins 5 autres espèces (McPherson, 2009). Il y a donc fort à parier que la majorité des espèces du genre soit viscoélastique et ce caractère semble par ailleurs plésiomorphe : la reconstruction par parcimonie sur l'arbre obtenu à partir des données ITS suggère que de l'état ancestral est « un liquide VE ». De plus ces propriétés sont vraisemblablement héritées d'un ancêtre commun avec les genres frères mais morphologiquement très différents de *Nepenthes* que sont *Drosera* et *Drosophyllum*, dont les feuilles à glue vraisemblablement aussi viscoélastiques sont également des pièges carnivores. L'absence de liquide viscoélastique chez certaines espèces correspondrait donc à une perte évolutive et cette perte se serait produite à plusieurs reprises, et indépendamment, au cours de l'évolution.

La présence d'une zone cireuse chez les urnes du haut et du bas, est également un caractère plésiomorphe. Alors que 4/5 des espèces présentent des urnes terrestres avec une zone cireuse, au moins 23 espèces perdent cette zone cireuse au cours de leur ontogénie. La perte ontogénétique de la zone cireuse s'est produite plusieurs fois et indépendamment au cours de la diversification du genre.

L'hétérophyllie ontogénétique et fonctionnelle mise en évidence par Laurence Gaume et Bruno Di Giusto sur *N. rafflesiana* n'est donc pas un cas unique et leurs hypothèses concernant le lien entre la forme et le fonctionnement du piège sont validées à l'échelle du genre : les espèces présentant une forme d'urne allongée sont quasi-exclusivement cireuses, celles dites infundibulées ou en forme d'entonnoir en sont quasiment toutes dépourvues. Les changements morphologiques marqués entre urnes du bas et urnes du haut sont d'ailleurs fortement associées à la perte de la zone cireuse.

Pressions de sélection à l'origine de la diversité fonctionnelle du genre

Si le lien entre la forme et la fonction des urnes est clairement établi, cela n'explique pas pour autant quelles pressions de sélection sont à l'origine de cette diversité morphofonctionnelle. Si une tendance émerge de la grossière et partielle représentation que nous pouvons seulement nous faire de l'histoire évolutive d'une lignée, alors on peut (peut-être) espérer tenir l'une des explications majeures de son évolution. Pour *Nepenthes*, l'absence d'un liquide viscoélastique est plus fréquente chez les espèces de plaine que chez les espèces de montagne (**Manuscrit II.2**), et les espèces où la zone cireuse est absente sont les plus viscoélastiques (**Manuscrit I.1**).

Tout d'abord, s'il parait évident que le piège est sous très forte sélection chez les plantes carnivores, il est plus délicat de déterminer pour un système de piégeage donné, par exemple pour les urnes de *Nepenthes*, quelles sont les pressions de sélection qui conduisent à la diversité observée. Plusieurs hypothèses non exclusives peuvent néanmoins être proposées. Premièrement, le liquide viscoélastique est très efficace pour capturer des insectes rampants et volants, alors que la cire est surtout efficace pour retenir dans l'urne des insectes terrestres. La proportion d'insectes volants semble plus importante dans l'entomofaune raréfiée des montagnes tropicales donc l'investissement dans un liquide viscoélastique plutôt que dans la cire s'avèrerait plus rentable pour la plante. Par ailleurs, une zone cireuse suffirait, quant à elle, à piéger les fourmis très abondantes dans les plaines tropicales et donc à satisfaire les besoins nutritifs de la plante.

Une perspective serait de tester s'il existe un lien entre la durée de vie des urnes et la viscoélasticité des urnes. Alors que le liquide s'acidifie au cours de la première semaine et que l'acidité semble maintenue ensuite tant que l'urne est fonctionnelle, il semble qu'un pool initial de polymères soit produit et fonctionnel au moment de l'ouverture de l'urne et que la viscoélasticité ne cesse de décroître au cours de la vie de l'urne (**Annexe 4**) et des urnes de plus d'une semaine auxquelles on substitue le liquide viscoélastique par de l'eau ne seront jamais plus très viscoélastiques (*obs. pers.*). Si ce patron est général, les urnes d'espèces viscoélastiques et fonctionnelles plus de 60 jours ne peuvent sans doute plus compter après cette période sur la moindre viscoélasticité de leur liquide. On peut donc s'attendre à ce que la plupart des urnes uniquement élastiques aient des durées de vie relativement courtes. De la même façon qu'il semble exister des stratégies architecturales *r* et *K*, il existe peut-être des stratégies différentes dans la combinaison durée de vie / fonctionnement des urnes. De la même façon, s'il est confirmé qu'il n'y a pas de « maintien » de la viscoélasticité au cours de la vie de l'urne, alors l'hypothèse précédemment émise d'une forte viscoélasticité initiale pour parer à la dilution par l'eau de pluie semble tenir (Gaume & Forterre, 2007).

D'une façon générale on peut s'attendre d'une part à ce que la spécialisation des stratégies de piégeage soit influencée par un mélange de facteurs biotiques et abiotiques, et d'autre part à ce qu'une description écologique plus subtile que la catégorisation binaire plaine/montagne permette de mieux comprendre cette spécialisation. En résumé, établir un lien causal qui dépasse la corrélation entre la structure de l'entomofaune et la sélection pour une stratégie donnée, est un défi que les tendances observées ici incitent à relever.

Ontogénie et phylogénie : un mécanisme évo-dévo pour expliquer ces diversités

Nous avons discuté des pressions de sélection et notamment de celles exercées par l'entomofaune, qui peuvent expliquer pourquoi nous observons la diversité fonctionnelle actuelle, mais nous n'avons pas encore proposé de mécanisme qui expliquerait comment cette diversité est apparue.

La labilité observée dans les changements de caractères suggère que la forme et le fonctionnement des urnes ne sont pas excessivement contraints phylogénétiquement. Par ailleurs, les changements de forme et pertes de la zone cireuse se sont produits plusieurs fois au cours de l'évolution du genre. Ceci suggère qu'un mécanisme développemental permet d'adapter la stratégie de piégeage aux pressions de sélection locales.

Nous proposons en conclusion un mécanisme évo-dévo qui explique, grâce aux reconstructions phylogénétiques et ontogénétiques, l'histoire et l'évolution développementale des caractères clés de la carnivorie. Les premières urnes post-cotylédonaires de toutes les espèces observées jusqu'ici sont viscoélastiques et cireuses. Ces caractères juvéniles et plésiomorphes peuvent être retenus ou perdus plus ou moins rapidement au cours de l'ontogénie et de la phylogénie. Ce mécanisme repose sur des hétérochronies, c'est-à-dire des changements dans la vitesse ou dans l'expression des séquences développementales. Pour le complexe *Nepenthes rafflesiana* qui a inspiré la plupart de ce travail de thèse, toutes les configurations de perte de cire existent : la variété *elongata* présente toujours des urnes cireuses, la variété *typica* perd sa zone cireuse lors que l'acquisition de la lianescence et les urnes du bas de la variété *gigantea* ne sont jamais cireuses. Si l'on se penche un peu sur les germinations, toutes les urnes des plantules sont cireuses. Chez *N. gracilis*, dont les urnes du bas sont cireuses mais pas élastiques, les toutes premières urnes sont cireuses et élastiques. Le cas le plus extrême est *N. ampullaria* et *N. bicalcarata* dont les caractères cireux et élastiques sont perdus très rapidement au cours de l'ontogénie, si bien que les urnes du bas de ces deux espèces ne sont jamais cireuses ni élastiques.

L'ontogénie ne récapitule pas la phylogénie dans le sens Haeckelien du terme mais la phylogénie permet en revanche de comprendre l'ontogénie, comment d'un ancêtre commun viscoélastique et cireux on passe à la diversité actuelle. L'embryogénèse végétale peut être suivie a posteriori le long d'un axe où les différents stades ontogénétiques sont exprimés. L'évo-devo est un champ disciplinaire relativement récent, historiquement dominé par les modèles animaux. Elle fournit à la biologie de l'évolution un éclairage puissant et élégant qui permet de comprendre comment de minimes changements dans le tempo et l'expression des séquences développementales conduisent à de profonds changements dans le phénotype exprimé. L'heure de gloire de l'évo-dévo est je pense devant nous et le genre *Nepenthes* en est un modèle de choix.

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ANNEXES

ANNEXE 1 : PUBLICATION

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The plant-ant *Camponotus schmitzi* helps its carnivorous host-plant *Nepenthes bicalcarata* to catch its prey

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Abstract: The Bornean climber, *Nepenthes bicalcarata,* is unique among plants because it is both carnivorous and myrmecophytic, bearing pitcher-shaped leaves and the ant *Camponotus schmitzi* within tendrils. We explored, in the forests of Brunei, the hypothesis that these ants contribute to plant nutrition by catching and digesting its prey. We first tested whether ants increased plant's capture rate. We found that unlike most plant-ants, *C. schmitzi* do not exhibit dissuasive leaf-patrolling behaviour (zero patrol on 67 pitchers of 10 plants) but lie concealed under pitcher rim (13 ± 6 ants per pitcher) allowing numerous insect visits. However, 47 out of 50 individuals of the largest visitor dropped into the pitchers of five plants were attacked by ants and the capture rate of the same pitchers deprived of their ambush hunting ants decreased three-fold. We then tested whether ants participated in plant's digestion. We showed in a 15-d long experiment that ants fed on prey and returned it in pieces in seven out of eight pitchers. The 40 prey deposited in ant-deprived pitchers remained intact indicating a weak digestive power of the fluid confirmed to be only weakly acidic (pH $\sim$5, n = 67). The analysis of 10 pitcher contents revealed that prey, mainly ants and termites, was very numerous ($\sim$400 per pitcher per plant) and highly fragmented. Altogether, these data suggest a positive effect of *C. schmitzi* on both prey intake and breakdown. This ant–plant interaction could thus be a nutritional mutualism involving the unusual association of carnivory and myrmecotrophy.

Key Words: Ant-plant mutualism, myrmecophyte, myrmecotrophy, pitcher plant, predation strategy

INTRODUCTION

Scarcity of essential nutrients has led to the evolution of alternative strategies of nutrition in plants, such as myrmecotrophy and carnivory, which allow them to obtain nutrients from animals (Juniper *et al.* 1989, Thompson 1981). Myrmecotrophy refers to ant-fed plants (Beattie 1989, Solano & Dejean 2004). Most myrmecotrophic plants are also myrmecophytes, i.e. plants that harbour ants in specialized cavities called domatia (McKey *et al.* 2005). These plants, often epiphytes, assimilate the decomposition products of the ant faeces and debris accumulated in modified stems, rhizomes or leaves (Huxley 1978, Janzen 1974, Treseder *et al.* 1995).

Carnivorous plants derive some key nutrients, such as nitrogen, from arthropods that they capture and digest in their traps (Ellison & Gotelli 2001, Juniper *et al.* 1989). The carnivorous genus *Nepenthes* comprises more than 100 species (Clarke 1997, McPherson 2009), mainly distributed in South-East Asia. Its traps are leaves modified as pitchers filled with an enzymatic fluid and a community of mostly dipteran and bacterial 'infauna' involved in the digestion process (Beaver 1983, Clarke & Kitching 1993, Cresswell 2000). The pitchers exhibit various combinations of characters involved in insect attraction and capture, such as UV patterns (Moran 1996) and sweet odours (Di Giusto *et al.* 2010, Moran 1996), wettable rims (Bauer *et al.* 2009, Bohn & Federle 2004), slippery waxy surfaces (Gaume & Di Giusto 2009, Gaume *et al.* 2002, Juniper *et al.* 1989) and viscoelastic digestive liquid (Di Giusto *et al.* 2008, Gaume & Forterre 2007). A few *Nepenthes* species have been shown to display unusual N-sequestration strategies, obtaining nitrogen from plant debris (Moran *et al.* 2003) or vertebrate faeces (Clarke *et al.* 2009).

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Nepenthes bicalcarata Hook. f., endemic to the peat-swamp forests of northern Borneo, is the only *Nepenthes* species known to be a myrmecophyte: it harbours in its tendrils the species-specific ant *Camponotus schmitzi* Stärke. In this study we explore the hypothesis that *N. bicalcarata* uses both carnivory and myrmecotrophy to circumvent nutrient scarcity.

This ant-plant association still remains intriguing. It was first proposed to be a mutualism in which the ants gain nectar and nest sites (Figure 1a–c) from their host-plant and confer on it some protection against pitcher putrefaction (Clarke & Kitching 1995). The authors reported that *C. schmitzi* ants were unaggressive but able to safely swim into the digestive liquid and remove large dead prey items, which paradoxically benefits the plant in avoiding sudden ammonium releases and subsequent pitcher putrefaction. In a later study, these ants were shown to aggressively defend their host-plant against a specific weevil that feeds on pitcher buds (Merbach *et al.* 2007).

These apparently two contradictory observations on the aggressiveness of *Camponotus schmitzi* towards insects raises the fundamental question of how these ants interact with insects visiting the mature and open pitchers for their nectar, hereafter called nectar visitors. Do they dissuade, as do most plant-ants, these visitors which are also potential prey of the pitcher plant or do they conversely facilitate their capture? Another not completely resolved question concerns their feeding behaviour and its impact on the plant's intake. To what extent do these ants consume the prey inside the pitcher and could they play a role in its breakdown and digestion by the plant?

The answers to these questions might help unravel the nature of the interaction between *C. schmitzi* and its host-plant and explore the hypothesis that the carnivorous plant obtains a nutritional benefit from its ant symbiont. To this end, we first tested the hypothesis that the ants hunt and help the plant to catch its prey, by quantifying the patrolling behaviour and aggressiveness of *C. schmitzi* towards pitcher visitors and fallen prey and by measuring their impact on prey capture using an ant-exclusion experiment. We then tested the hypothesis that they regularly consume part of the prey and help in the plant's digestion. Using a 15-d long prey-deposition experiment, we studied the frequency of their feeding behaviour towards experimental prey of two size-categories and its impact on prey breakdown. We also analysed the prey contents of pitchers and measured the pH of the fluid, to obtain data respectively on the plant's prey intake and on its digestive ability.

STUDY SPECIES AND STUDY SITE

Nepenthes bicalcarata is a liana that climbs up to 20 m and has enormous leaves with pitchers that are long-lived in comparison to other *Nepenthes* species (Cheek & Jebb 2001, Clarke 1997). The tendril that sustains its trap is swollen (Figure 1a) and inhabited by the small ant *Camponotus schmitzi* (Formicinae), that has never been reported living outside its host-plant (Clarke & Kitching 1995, Jolivet 1986). The most characteristic structures of *N. bicalcarata* are the two giant nectaries shaped like thorns that overhang the pitcher's mouth (Figure 1b) and are exploited by *C. schmitzi* and other ants (Merbach *et al.* 1999).

All experiments were carried out in July–August 2009 in a mixed peat swamp and heath forest (4°44′N, 114°35′E) of Brunei Darussalam (northern Borneo) on *N. bicalcarata* upper pitchers that were all occupied by *C. schmitzi*.

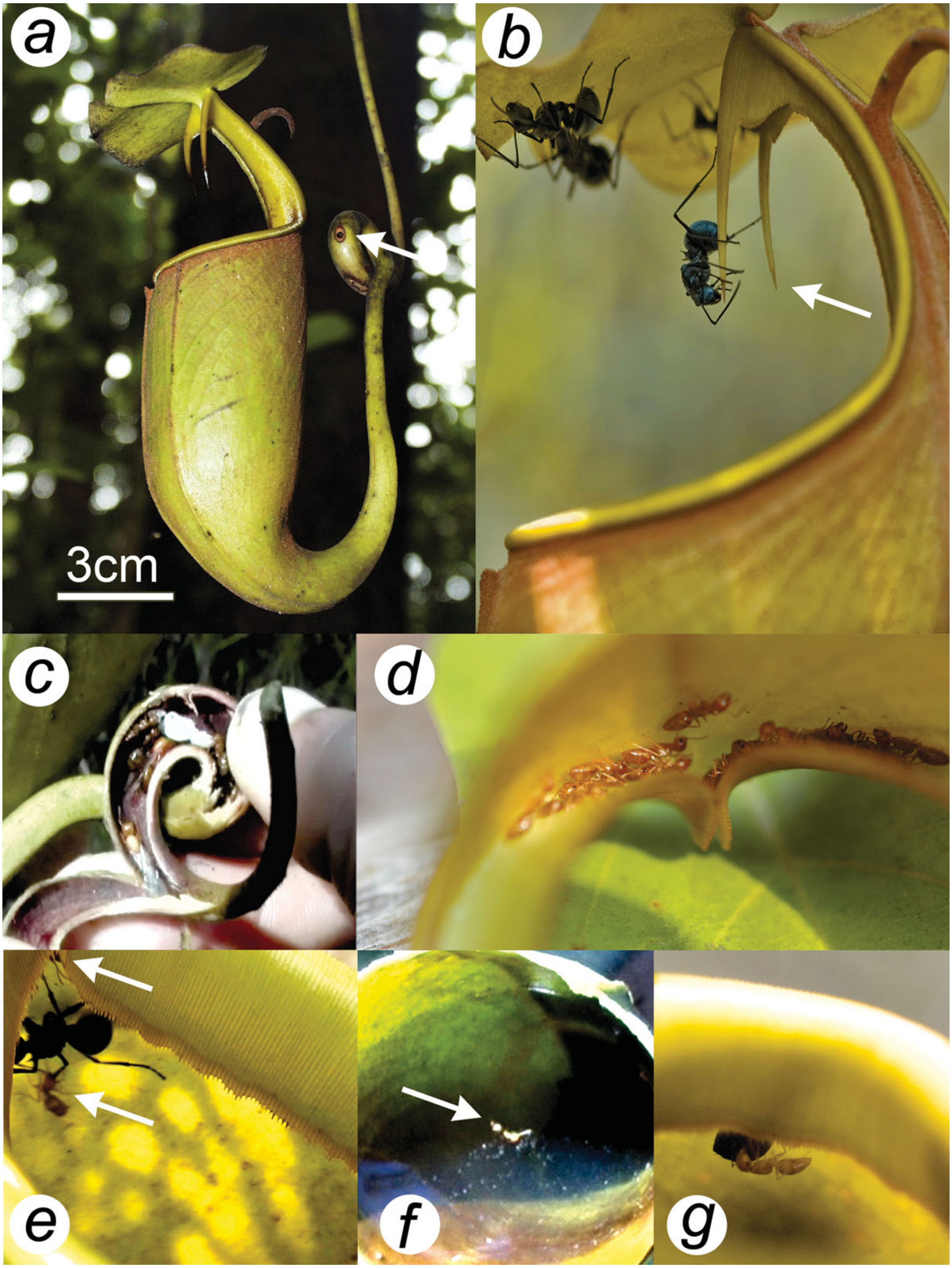
METHODS

Measure of ant territoriality and pitcher fluid acidity

To assess the territoriality and patrolling behaviour of *C. schmitzi* outside the pitchers, we counted over 1 min the total number of *C. schmitzi* workers and arthropod visitors on nectariferous external parts of the pitchers on a total of 67 functional pitchers of different age classes belonging to 10 plants. All observations were made around 10h00 in sunny conditions over 10 d, i.e. in rather homogeneous conditions of nectar production and insect activity. After each observation, the pH of pitcher fluid was measured using pH-indicator strips (Acilit® pH 0–6.0, Merck Chemicals, Darmstadt, Germany). We wished to examine on a large sample, representative of the different age-classes of pitchers, if the pH of this fluid was not very acidic as already observed by Clarke & Kitching (1993) and if such a low acidity was maintained throughout the pitcher life span. Constant neutral pH or moderate acidity would mean that the fluid is inoffensive towards the swimming ant symbiont but also not very efficient in dissolving the prey.

Insect retention experiment

We then tested the aggressiveness of *C. schmitzi* towards other insects inside the pitchers and tested their effect on prey retention. We selected, as experimental prey, ants commonly found feeding on the extrafloral nectaries of *N. bicalcarata*. We first tried to use a small-bodied ant species (*Crematogaster* sp. 1, total length = 3.5 mm, Table 1), one of the two most common visitors and prey of *N. bicalcarata*, but workers of this species were never observed to escape from the digestive liquid even when the pitchers were deprived of their *C. schmitzi* ants. Hence they could not be good candidates to test



COLOUR

Figure 1. Overview of the ant–plant interaction. Upper pitcher of *Nepenthes bicalcarata*. The arrow indicates the domatium within the coiled tendril which harbours *Camponotus schmitzi* ants (a). *Polyrhachis pruinosa* workers feeding on the extrafloral nectar produced by the pitcher on the inner side of the lid and by the two thorns (arrow) surrounding the pitcher (b). Sectioned domatium showing workers, alates and brood of *Camponotus schmitzi* (c). *Camponotus schmitzi* workers in ambush position under the peristome (d). A fallen *Polyrhachis pruinosa* ant being attacked (arrows) by two *Camponotus schmitzi* (e). *Camponotus schmitzi* (arrow) can safely swim in the digestive liquid from which it removes prey items (f) that are then consumed under the peristome (g).

Table 1. Prey composition of 10 pitchers of *Nepenthes bicalcarata* from a mixed peat swamp and heath forest of Brunei Darussalam (Northern Borneo).

Prey contents of *N. bicalcarata* pitchers	Mean ± SD number of individuals per pitcher	Prevalence (% of pitchers where the taxon was present)
Hymenoptera		
Ants (Formicidae)		
Formicinae		
Camponotus schmitzi (queen + worker)	6.1 ± 4.7	100
Camponotus sp.	5.2 ± 7.9	50
Polyrhachis pruinosa	1.5 ± 1.1	90
Polyrhachis sp. 1	1 ± 1.1	60
Polyrhachis sp. 2	0.3 ± 0.5	30
Anoplolepis gracilipes	0.1 ± 0.1	10
Myrmicinae		
Crematogaster sp. 1	27.2 ± 39.2	80
Crematogaster sp. 2	10.7 ± 14.8	60
Pheidole sp.	14.8 ± 41.2	20
Myrmicinae sp. 1	1.6 ± 5.1	10
Dolichoderinae		
Tapinoma sp. 1	25.7 ± 81.3	10
Tapinoma sp. 2	15.1 ± 35.5	20
Dolichoderinae sp. 2	8.6 ± 16.4	30
Unidentified ants	1.4 ± 2.3	40
Total ants	119 ± 123.1	100
Apidae sp.	0.2 ± 0.4	20
Vespidae sp.	1.0 ± 2.2	30
Total Hymenoptera	120 ± 124	100
Total Lepidoptera (larva)	0.1 ± 0.3	10
Total Diptera	2.9 ± 3.0	80
Isoptera		
Termidae (Nasutitermitinae)		
Hospitalitermes sp. soldier caste 1	118 ± 207	40
Hospitalitermes sp. soldier caste 2	53.8 ± 169	20
Hospitalitermes sp. worker caste	141 ± 219	40
Total Isoptera	312 ± 412	40
Total Coleoptera	2.2 ± 2.0	80
Araneae (Salticidae sp.)	0.2 ± 0.4	20
Total prey	438 ± 374	100

whether *C. schmitzi* deployed aggressiveness toward fallen prey. We thus chose *Polyrhachis pruinosa* Mayr, another common visitor and prey item of the plant (total length = 1.2 cm, Table 1, Figure 1b), as they were easier to handle and they usually succeeded in escaping from the liquid.

Five *C. schmitzi*-occupied pitchers belonging to five different plants were haphazardly selected. A *Polyrhachis* ant was dropped into the digestive liquid of each pitcher, and the fate (retained/escaped) and behaviour (time needed to escape from the pitcher, the number of times the ant fell back into the liquid) of this ant were observed and sometimes video-recorded. To drop the ant into the pitcher's digestive liquid, we first drew it into a soft tube and then blew it onto the digestive liquid without direct manipulation. We repeated this experiment with 10 different ants for each pitcher. Intervals between successive trials were less than 5 min when the ant succeeded in escaping within the 5 min. When the ant did not escape within 5 min, we observed it for more than 20 min, to check that it was effectively killed. The ant was then removed from the pitcher before the next trial. The results of this experiment were subsequently compared with the fate and behaviour of 10 *Polyrhachis* ants on the same pitchers but deprived of *C. schmitzi*. We used smoke produced by burning dead leaves to deter the latter. Twenty trials were thus conducted per pitcher (10 trials with and 10 trials without *C. schmitzi*). An ant was considered as retained if it did not escape within 5 min. The ants that did not successfully escape within 5 min were observed to die, except for two ants that were observed to be exhausted, exhibiting very slow movements.

Data were analysed using the software package SAS v.9.1. We tested for an effect of *C. schmitzi* (fixed factor) and pitcher (random factor) on the percentage of ants trapped using a mixed logistic regression by using the macro GLIMMIX with a binomial error distribution. We tested for an effect of *C. schmitzi* (fixed factor) and pitcher (random factor) on the number of times the experimental ant slid back into the digestive fluid after an escape attempt with a mixed Poisson regression model by using the macro GLIMMIX with a Poisson error distribution. Correction for over-dispersion was applied using the square root of the ratio of Pearson's χ^2 to the associated number of degrees of freedom. We tested for an effect of *C. schmitzi* (fixed factor) and pitcher (random factor) on the time required to escape, by using a mixed-model analysis of variance with the GLM procedure. For model selection backward procedures were adopted, starting with the removal of non-significant interactions.

Feeding behaviour of *Camponotus schmitzi*

A second experiment investigated the feeding behaviour of *C. schmitzi* towards deposited prey of two different sizes and comparable to the sizes of the prey we observed inside the pitchers of *N. bicalcarata*. Ten upper pitchers were haphazardly selected, each belonging to a different plant. Their arthropod contents were removed by filtering the liquid with a mesh and the digestive liquid was then returned to the pitchers. Tanglefoot© insect glue was applied on the leaves bearing the pitchers and on the vegetation in contact with the tendril so that no crawling insects other than *C. schmitzi* could reach the prey items. In each of the 10 arthropod-free pitchers, we introduced 10 entire bodies of a *Crematogaster* species and 10 gasters (abdomens) of *Polyrhachis pruinosa*. Neither of these prey items could be mistaken for any other insect that could have fallen – despite our precautions – into the pitchers. Firstly, the *Crematogaster* species used in this experiment comes from another site and was never observed in the site where the experiment was conducted. Secondly, we only used gasters of *Polyrhachis pruinosa* to be sure that they belong to our experimental prey items and not to the bodies of possibly newly fallen workers of this species, common in this site. The two prey items measured respectively 2 mm and 5 mm, both within the main size range of prey of *Nepenthes bicalcarata*. *Camponotus schmitzi* ants were removed from two of these 10 pitchers, as well as from their associated domatium. These two pitchers were used as controls to check that in the absence of *C. schmitzi*, no prey was removed from pitchers. Fifteen days later, the content of each of these 10 pitchers was collected, counted and observed using a binocular microscope. As the total numbers of experimental prey items were equal (=10) for each pitcher and each prey category, we tested for an effect of prey category (fixed factor) and pitcher (random factor) on the number of items presenting obvious signs of ant predation by using a mixed Poisson regression model with the macro GLIMMIX specifying a Poisson error distribution.

Analysis of prey contents

The prey contents of 10 old but still functional pitchers (the pitcher borne by the node immediately below being senescent), all inhabited by *Camponotus schmitzi* and belonging to different individuals were collected in the same site in September 2008 in 10 vials filled with 70% alcohol, then analysed and counted in the laboratory using a binocular microscope. The necromass was composed of plant and animal parts as well as of small 'pellets' of fine particles that might be the faeces of *C. schmitzi* or of mosquito larvae, although we never observed such kinds of faeces in the pitcher fluid of other *Nepenthes* species that all bore mosquito larvae. Only the animal parts were considered in this analysis. The prey items found in these pitchers were highly disintegrated compared to those in other *Nepenthes* species. Almost no entire bodies were present, but only pieces of exoskeleton. Identification was often only possible by comparing the remains of head capsules and body parts with insects collected alive on the nectariferous parts of the pitcher plant. The dipterans were not sufficiently well preserved to be identified further than at the order level and this was often true for the coleopterans, as well. Only the ants were distinguished to morphospecies and identified to genus, when possible, using the identification key of Hölldobler & Wilson (1990).

RESULTS

Absence of dissuasive patrols of *Camponotus schmitzi* towards nectar visitors

We observed on average 4.8 ± 3.8 (mean ± SD given hereafter, on 67 pitchers), nectar visitors (4.5 ± 3.9 ants and 0.3 ± 1.0 flying insects) on the outer part of pitchers during each observation. Most of these arthropods, including ants (belonging to the genera *Crematogaster* (*Crematogaster* sp. 1: 2.4 ± 3.7), *Polyrhachis* (*Polyrhachis pruinosa*: 1 ± 1.5), *Oecophylla*, *Pheidole*, *Anoplolepis* and *Tapinoma*), midges, mosquitoes and lepidopterans were found feeding on nectar of the lower face of the lid (Figure 1b). By contrast, no *C. schmitzi* workers were found walking on the pitcher body, although these ants were present on each of the pitchers observed, hidden under the peristome all around its edges (13.2 ± 6.3 workers, ants counted on a subset of 10 out of the 67 pitchers, Figure 1d). The digestive liquid had a mean pH of 4.9 which was rather constant despite pitcher age differences (SE = 0.05, N = 67 pitchers).

Effect of *Camponotus schmitzi* on ant retention

Camponotus schmitzi-occupied pitchers retained about three times more *Polyrhachis pruinosa* ants (mean = 26%, SE = 6.8%, 50 ants) than did the same pitchers when deprived of their symbiotic ant (mean = 8%, SE = 3.7%, 50 ants), as shown by the logistic regression (fixed effect of the *C. schmitzi* treatment: $F_{1,98} = 4.7$, P = 0.03, no random effect of pitcher: variance estimate = 0.08 vs. residual = 1.08).

Indeed, when *Polyrhachis pruinosa* ants attempted to escape from the digestive liquid, they were in almost all cases (47 ants out of 50) attacked by *C. schmitzi*, which ambushed them from under the peristome. We regularly observed one or several *C. schmitzi* ants biting the legs of *Polyrhachis* ants. The small *C. schmitzi* ants mainly attack the intruders from the peristome, the curved and sharp teeth of which form a protective shield, but they sometimes also leave their refuge to attack the intruder from underlying parts of the pitcher (Figure 1e, on-line video clip, http://umramap.cirad.fr/amap3/cm/index.php?page = films). As a consequence of these attacks, the *Polyrhachis* ants slipped more often in *C. schmitzi*-occupied than in *C. schmitzi*-deprived pitchers, in which they only had to cope with the slipperiness of the pitcher walls and with the previous lubrication of their tarsal pads by the digestive fluid (Poisson regression on number of falls: $F_{1,98} = 15.8$,

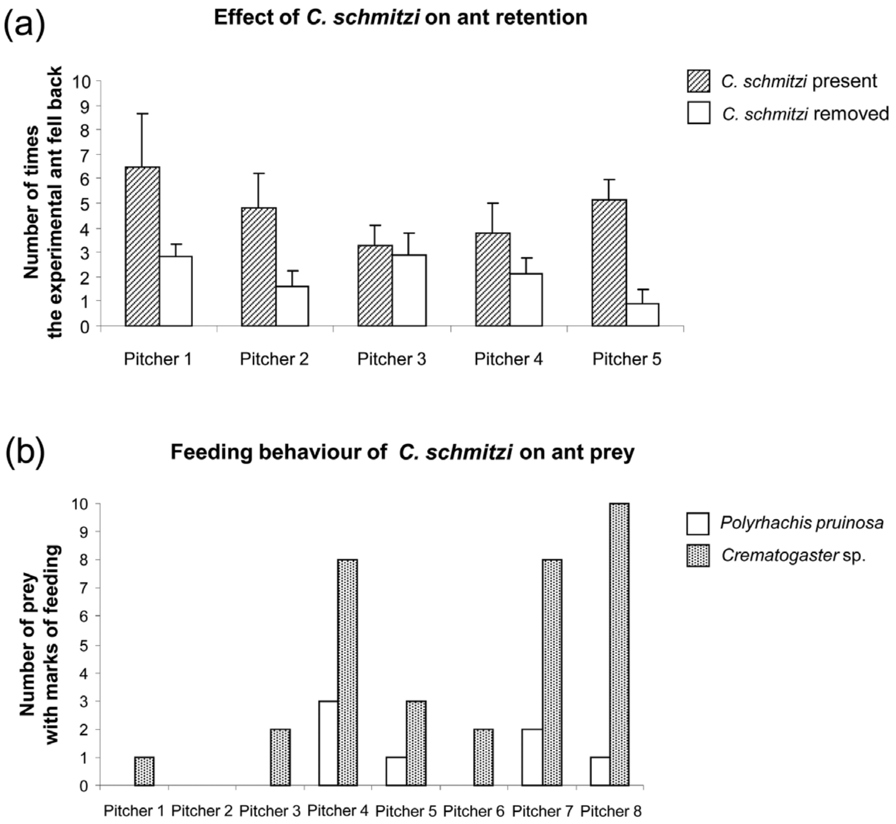


Figure 2. Experimental results showing the effect of the ant symbiont on both prey retention and breakdown in *Nepenthes bicalcarata*. Effect of *Camponotus schmitzi* on ant retention (a). The x-axis refers to the different experimental pitchers. The y-axis refers to the number of times the experimental ant fell back into the digestive liquid, attempting to escape from the pitcher, during a 5-min session. The error bars refer to 1 SE. Evidence of feeding behaviour of *Camponotus schmitzi* on dead ants of two different sizes (b). The x-axis refers to different experimental pitchers. The y-axis refers to the number of prey (out of 10) which bore marks of ant feeding and breakdown after the 15-d experiment. No prey breakdown was observed in the control pitchers (not shown) where *C. schmitzi* was excluded.

P < 0.0001; no random effect of pitcher: variance estimate = 0.001 vs. residual = 3.08, Figure 2a). The ants that did not escape from the digestive liquid usually died from exhaustion and drowning following numerous unsuccessful attempts to escape from the pitcher and/or numerous bites from *C. schmitzi*. Furthermore, when the *Polyrhachis* ants succeeded in escaping the pitchers within the 5-min observation time, the time required for them to escape was significantly longer in the presence of *C. schmitzi* ants (mean ± SD = 127 ± 75 s, range 12–282 s, N = 37) than when these ants were absent (83 ± 64 s, range 3–250 s, N = 46). Time required to escape also significantly varied among pitchers (mixed-model ANOVA on time to escape: fixed effect of *C. schmitzi* treatment: $F_{1,77} = 9.01$, P = 0.004; random effect of pitcher: $F_{4,77} = 3.52$, P = 0.01). *Polyrhachis pruinosa* used in the bioassays, one of the two most common prey species, was also the largest prey item recorded in the analysis of prey contents and probably the hardest to catch for *C. schmitzi*. Hence our results probably underestimated the effect of *C. schmitzi* on prey retention and our tests are therefore conservative in that respect.

Feeding behaviour of *Camponotus schmitzi* towards dead prey in the pitchers

Camponotus schmitzi were occasionally observed to swim in the digestive liquid and pull an ant prey item, such as *Crematogaster* or *Polyrhachis*, out of the liquid. They then hauled it up to the underside of the peristome and consumed part of the dead insect, dropping into the pitcher the cuticular remains and other uneaten parts (Figure 1f, g). No flying insect was observed to be trapped in any of the 10 pitchers during the 15-d experiment.

Thus in the presence of *C. schmitzi*, on average 42.5% of the experimental *Crematogaster* ants were either entirely removed from the liquid (1 *Crematogaster* ant found underneath the peristome) or presented traces of mandibles on the remaining body parts (34 ants, SE = 13.5), and 8.7% of the *Polyrhachis* ants (7 ants, SE = 4). Such dismemberments could not have resulted from plant digestion, since none of the 40 ants in the control pitchers deprived of *C. schmitzi* showed any sign of such dismemberment. Moreover, flying insects constituted only 6% of the nectar visitors and less than 1% of the prey, and we never observed any flying insects feeding on prey in the pitcher. As crawling insects were excluded from the experiment by the glue, the feeding activity on dead prey was attributed to *C. schmitzi*. Our data indicate that *C. schmitzi* fed preferentially on the small *Crematogaster* prey items (parts or entire bodies) rather than on the bigger *Polyrhachis* prey items and that there was globally more such feeding activity in some pitchers than in others (Poisson regression on number of insect items with signs of ant feeding: fixed effect of ant species: $F_{1,7} = 35.9$, $P = 0.0005$, Figure 2b; random effect of pitcher: variance estimate = 1.39 vs. residual = 0.40).

Analysis of the *Nepenthes bicalcarata* prey spectrum

Ants were found in 100% of the traps, with up to 386 individuals per pitcher (Table 1). Among them, 13 morphospecies were identified. The most frequently represented ant species included *Camponotus schmitzi* itself (remaining heads of queens or workers were found in 100% of the pitchers), *Polyrhachis pruinosa* (small numbers of individuals found in 90% of the pitchers) and *Crematogaster* sp. 1 (in 80% of the pitchers and up to 124 individuals found in a pitcher). Large numbers of small myrmicines were more occasionally observed in the pitchers. Large numbers of termites (up to 946 per pitcher) of a *Hospitalitermes* species were found in 40% of the pitchers. Ants and termites thus constituted 98% of the prey of *N. bicalcarata*, the flies and midges 1% and the beetles 0.5% (Table 1). Hundreds of minute pellets, never found in the pitchers of other *Nepenthes* species we analysed (unpubl. data) were found in each pitcher.

DISCUSSION

Our behavioural observations and our experiments showed that *Camponotus schmitzi* participates in the process of prey capture by its host-plant, *Nepenthes bicalcarata*, and may also contribute to the process of prey digestion. This plant-ant systematically attacks trapped visitors that attempt to escape from the pitcher and scavenges on dead prey items that it collects from the digestive liquid of its carnivorous host-plant. It consumes part of the prey within the pitcher under the peristome and returns to the digestive liquid substantial non-eaten pieces and maybe also its faeces.

Here, we report new data that may reconcile the apparently conflicting observations reported by Clarke & Kitching (1995) and Merbach *et al.* (2007) concerning the behaviour of *C. schmitzi* towards insects: the ants combine times of passivity and aggressiveness which seems to be part of a unique adaptive strategy. On one hand, the *C. schmitzi* ants were shown, at least during daytime, to be unaggressive on open, nectar-producing mature pitchers. Such an absence of territoriality is very unusual for plant-ants, which often exhibit specialized behaviours, such as systematically patrolling nectar-producing sites which dissuades intruders including herbivorous insects (Gaume *et al.* 2005, 2006; McKey *et al.* 2005), or such as pruning their host-plant neighbours to avoid competition from other ants (Federle *et al.* 2002). On the contrary, *Camponotus schmitzi* ants conceal themselves and allow numerous insects, mainly other ants, to visit and feed on pitcher nectar. Conspicuous activity of the ants would deter potential prey, reducing benefits both to the carnivorous host-plant and to its ant colony. On the other hand, our data provide further evidence that aggressiveness can be part of the behaviour of *C. schmitzi* ants but that the ants display a 'delayed' aggressiveness which only occurs once the visitors fall into the trap. Attacks by this ant prevent insects from escaping the pitcher and its experimental exclusion decreases the prey retention rate of its host-plant. Hunting by ambush from the shelter of the curled lip of the peristome, this ant has also escaped the attention of most observers. The often unnoticeable aggressiveness that they deploy from this shelter might explain why in other studies, insects attempting to escape from the pitcher had so much difficulty in crossing the peristome (Bohn & Federle 2004). The behaviour of *C. schmitzi* is quite similar to the ambushing behaviour of a tiny Amazonian myrmicine *Allomerus decemarticulatus*, which constructs holed platforms on its host-plant, *Hirtella physophora*, to catch and kill large insects (Dejean *et al.* 2005). Complementary experiments with other prey species should provide further information on the effect of *C. schmitzi* on its host-plant efficiency at capturing diverse prey.

Although the reciprocal effects on fitness of the two partners will not be easy to demonstrate experimentally, mainly because long-term exclusion of the ants would also imply an exclusion of potential crawling prey, we provide here several results which support the hypothesis of a nutritional mutualism between *N. bicalcarata* and its ant. Our results clarify the benefits of the interaction for the ants. In addition to nesting structures and sugar-rich nectar offered by the plant, our results show that the diet of *C. schmitzi* includes a regular protein meal obtained from plant-trapped prey. Indeed, the ants not only feed on large prey items, as already observed by Clarke & Kitching (1995) but also (and probably more often) on smaller ones, as supported by our insect bioassays. Although the retention experiments focused on one type of prey – the biggest and probably the most difficult to trap – our results also suggest that the plant benefits from a food surplus provided by the symbiotic ants through their hunting behaviour. The ants only eat a small part of the prey and drop the non-metabolized parts into the pitchers. Furthermore, their activity of prey-breakdown and their metabolic activity should facilitate nutrient assimilation by the plant as does the living infauna of the digestive liquid (Beaver 1983).

It is probably not a coincidence that ants and termites were found to be abundant and numerically dominant among the prey items of *N. bicalcarata.* Indeed, they are typically the kind of prey that can be easily targeted by *C. schmitzi*, which is probably much less effective against flying insects, which do not need to climb on the inner wall or on the slippery peristome to escape from the pitcher. Termites of the genus *Hospitalitermes* (Nasutitermitinae) are nocturnally active processional termites (Jones & Gathorne-Hardy 1995). This is probably why we did not record them as pitcher visitors. They forage in large numbers and climb at night on the surrounding vegetation in search of food. Since they are virtually blind, they should be attracted by the odour cues of the pitchers, probably as in *N. albomarginata* (Merbach *et al.* 2002). However, because of their nomadic way of life, they are likely to be less reliable prey than ants, although termites have been recorded in the pitchers of *N. bicalcarata* (Cresswell 2000). Since ants, the most consistently reliable prey of *N. bicalcarata*, have been estimated to provide 70% of the nitrogen used in several *Nepenthes* species (Moran *et al.* 2001, Schultze *et al.* 1997), the additional proteins provided to the plant by the hunting behaviour of *C. schmitzi* should represent a fitness advantage for the host-plant in the nutrient-poor environments in which it occurs. In addition, the plant could probably benefit from direct myrmecotrophy since dead bodies of *C. schmitzi* were systematically found in the pitcher contents (Table 1). Because these ants rarely leave their pitcher refuge, their faeces are likely dropped into the digestive liquid and could constitute another source of nutrients assimilable by the plant. However, the ant-origin of the observed pellets in the digestive fluid remains to be demonstrated by further experiments and its participation to the plant nutrition needs to be demonstrated by isotopic analyses. Added to the anti-herbivore (Merbach *et al.* 2007) and anti-putrefaction (Clarke & Kitching 1995) benefits mediated by the ant, this nutritional benefit provided by the ant supports the hypothesis of a multi-faceted mutualism between this symbiotic ant and its carnivorous host-plant.

The association with a hunting plant-ant also constitutes a novel specific trapping strategy in the genus *Nepenthes*, which already displays a large spectrum of insect-trapping devices. The peristome was demonstrated to be very wettable in this species and to be the major surface responsible for insect fall due to aquaplaning following rain or nectar secretion (Bohn & Federle 2004). We never saw any nectar spreading over the peristome of *N. bicalcarata* during our study time, contrary to other *Nepenthes* species such as *N. rafflesiana*, but we showed that the concealed ants play a crucial role in preventing the ascent of the peristome of *N. bicalcarata* by trapped insects. Moreover, the association with a hunting ant makes sense in relation to the absence in *N. bicalcarata* of costly and efficient trapping features such as a slippery waxy layer made of long-chain aldehydes (Gaume & Di Giusto 2009), or a viscoelastic fluid made of giant polymers (Gaume & Forterre 2007).

We confirm the results reported by Clarke & Kitching (1993) on a smaller sample of pitchers that the digestive fluid of *N. bicalcarata* is less acidic than the fluid of several of its congeners, such as *N. rafflesiana* (Bauer *et al.* 2009), *N. alata* (An *et al.* 2002) or *N. gracilis* (Clarke 1997), all of which have been shown to have a pH close to 2.5, optimal for the activity of nepenthesin proteases (Athauda *et al.* 2004). This elevated pH might have an adaptive significance and protect the plant-ant symbiont (Moran *et al.* 2010) but it also suggests that *N. bicalcarata* might not have the same pool of enzymes as the other *Nepenthes*. As *N. bicalcarata* has one of the richest and most diverse aquatic infauna in the genus (Clarke & Kitching 1993, Cresswell 2000), it might be heavily dependent on its infauna, and on *C. schmitzi*, to digest its prey. The additional work of pre-digestion by *C. schmitzi* could also compensate for a potentially weak digestive efficiency of the plant itself, due to the low acidity of its digestive fluid. Furthermore, the very long life span of *N. bicalcarata* pitchers and the plants' large size (Clarke 1997) are undoubtedly advantageous for their ant inhabitants, as they provide reliable nest-site and food resources for these permanent residents. It is tempting to hypothesize that these traits have evolved in the context of coevolution between the two partners. Long-term experiments need to be carried out to test these hypotheses and to assess whether the ant-plant mutualism is the outcome of reciprocal adaptation of the two partners.

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ANNEXE 2 : MESURER LA VISCOÉLASTICITÉ, UNE MÉTHODE D'ANALYSE

Quantifier la viscoélasticité n'est pas chose aisée. Diverses techniques existent mais elles ne sont généralement pas adaptées à de petites quantités de fluide peu élastique (Lakes, 2004). La méthode que nous avons retenue est la rhéométrie par rupture de filament (Rodd *et al.*, 2005). L'idée est de mesurer la dynamique de rétrécissement d'un filament du fluide et/ou le temps de vie du filament crée par une goutte du liquide que l'on veut mesurer préalablement étiré entre deux points. Cette méthode, déjà appliquée avec succès pour le fluide digestif des *Nepenthes* (Gaume & Forterre, 2007) a l'avantage de couvrir la gamme entière des temps de relaxation observés au sein du genre, de quelques centièmes de secondes pour les fluides non-élastiques ou très faiblement jusqu'à plusieurs secondes pour les fluides les plus élastiques.

La dynamique de rétrécissement du filament est délicate à mesurer car le temps de vie du filament peut être très court, de l'ordre d'une dizaine de millisecondes. Pour de si faibles élasticité, le même liquide étiré entre deux doigts ne sera alors pas diagnostiqué à l'œil nu comme viscoélastique. Par ailleurs, la méthode employée utilise une caméra ultra-rapide capable de filmer à une résolution convenable (600 x 400 pixels) à 2000 images par secondes. Un appareil conçu spécifiquement étire une goutte de liquide d'environ 40 µl entre deux plots, dont le supérieur s'écarte d'une distance fixe à l'aide d'un électroaimant commandé par l'utilisateur. Un rétroéclairage à diodes électroluminescentes procure des images contrastées des marges externes du filament, dont les plans orthogonaux à l'éclairage et l'optique apparaissent contrastés (idéalement noirs sur blanc) par le jeu des réfractions sur la section cylindrique du filament. Cet éclairage puissant fournit suffisamment de lumière pour que la succession d'images prise avec un temps d'exposition (l'inverse de la fréquence d'échantillonnage) parfois inférieur à 1 ms soit suffisamment exposées.

Pour quelques films, il est envisageable d'extraire manuellement le temps de vie du filament, défini comme le temps entre la fin de l'étirement du plot supérieur et la rupture de ce filament. Pour l'analyse répétée de nombreux fluides, la quantité de films à analyser, et surtout le nombre d'images, d'autant plus grand (à fréquence d'échantillonnage égale) que les fluides sont élastiques, devient rapidement inenvisageable (plusieurs dizaines de milliers ou centaines de milliers d'images). Il fallait donc automatiser ce processus et la solution retenue, qui pourra faire l'objet d'une distribution ultérieure, est un script d'analyse couplé entre ImageJ pour la rapidité du traitement des images et R pour la manipulation de fichiers, la visualisation et la facilité de programmation (Abramoff *et al.*, 2004, R Development Core Team, 2009).

Le principe d'analyse, détaillé dans la Figure 1, est le suivant :

i) on dispose pour chaque échantillon d'un film ;
ii) on ajuste l'image pour une détection optimale du filament ;
iii) l'utilisateur définit une zone d'intérêt correspondant au point médian entre les deux plots qui s'écartent ;
iv) iii) ImageJ décompose chaque vidéo en une série d'images et fournit pour chaque colonne de pixel de la zone d'intérêt la moyenne des niveaux de gris (compris entre 0 et 255) ;
v) ImageJ écrit pour chaque vidéo un fichier texte de (nombres d'images x nombre de colonnes) lignes correspondant à autant de valeurs de niveaux de gris ;
vi) R récupère les fichiers texte ;
vii) R seuille pour chaque image le profil des niveaux de gris et le transforme en signal carré ;
viii) R renvoie, pour chaque image, la distance maximale entre deux valeurs égales à 1, ce qui correspond au diamètre du filament ;
ix) On procède à une détection des artefacts de mesures (oscillations du filament, remontée de gouttes le long du filament, etc.) à l'aide d'une variance mobile et on supprime ces points ;
x) On procède à une vérification manuelle du profil obtenu, s'il correspond à une dynamique viscoélastique ;
xi) A la fin de toutes les analyses, R écrit un fichier .pdf par film et renvoie les résultats : viscoélasticité (0/1), temps de vie mesuré du filament, temps nécessaires pour que les diamètres soient réduits à 1/2, 1/5, 1/10, 1/n, pour chacun des films.

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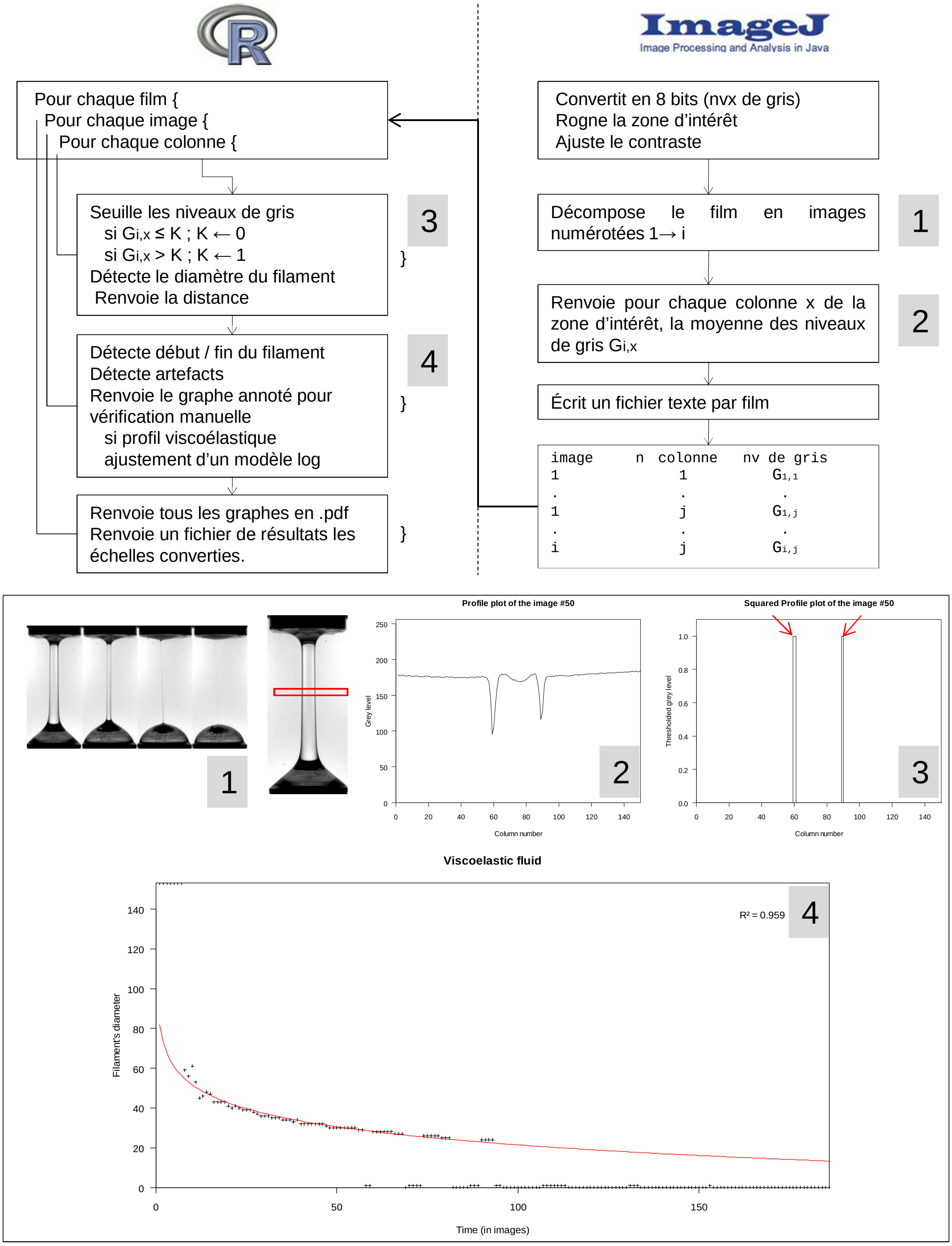

Figure 1 : Schéma illustré du fonctionnement du script d'analyse R-ImageJ de liquides viscoélastiques.

ANNEXE 3: DEVELOPMENTAL CHANGES OF GROWTH, PHOTOSYNTHETIC AND CARNIVOROUS COMPARTMENTS OF FIVE LOWLAND *NEPENTHES* CARNIVOROUS PLANTS

Vincent Bonhomme, Bruno Di Giusto, Isabelle Gounand,
Daniel Barthélémy and Laurence Gaume

During our field expeditions (2008, 2009) we conducted a growth survey and a node by node description of five lowland species. Data collection for this study will end next year but we nevertheless present the aims of this study and the first results, presented as graphics and briefly discussed.

Aims of this study

The aim of this study is to provide the first architectural description of five lowland *Nepenthes* with a special emphasis on the dynamics of the vegetative growth, flowering activity of the photosynthetic and in the carnivorous compartments and theirs eventual changes throughout ontogeny. Architectural concepts and analysis methods provide a powerful tool for studying the dynamics of plants' traits and ontogeny (Barthélémy & Caraglio, 2007). Among the questions related to this thesis our data should allow to investigate the following points:

- On the basis of simple traits such as growing rates, photosynthetic area, number of functional pitchers and their lifespan can we describe different ecological strategies? Since some species produce large and long-lived pitchers (e.g. *N. bicalcarata*) others small and short-living pitchers (e.g. *N. gracilis*), it seems that some *r* and *K* strategies exist for the pitcher production (Clarke, 1997). Can we characterize similar patterns for the photosynthetic compartment and more widely how much do these traits vary between species and do they define specific strategies?

- Within each species, do these characters change between the self-supporting stage and the climbing stage? In particular, what constraints exert the climbing habit on the leaf and carnivorous traits? Whereas they are produced almost systematically and lay on the ground during the self-supporting stage, do the number of pitchers is lower when the plants have to hang on surrounding vegetation? Similarly, do the leaf lengths is reduced and/or the geometry changed during the climbing stage? By the way, can we obtain reliable leaf allometries so that we can get a quick and good estimation of leaf area by measuring its length?

- Is there a marked seasonality in the flowering and growth phenologies? Can we estimate the age of the plants by their architectural description i.e. can we identify reliable markers such as inflorescence scars or internode sequence to redraw their development?

Methods and first results

All measurements have been done in July-August 2008 and in July-August 2009 in Brunei Darussalam (northern Borneo) in a mixed peatswamp heath forest (Labi Road) and in coastal white sands (Tutong). We described the five following lowland species: *N. bicalcarata*, *N. rafflesiana* var. *elongate* and *N. rafflesiana* var. *gigantea* in a mixed peatswamp and heath forest and *N. gracilis* and *N. rafflesiana* var. *typica* in coastal white sands (Tutong).

Three different ontogenic stages were defined: ‘self-supporting’, ‘transitional’ and ‘climbing’. Transitional stages were simply defined as intermediate between entirely self-supporting plants and entirely climbing plants, i.e. when the main axis was already hang but when most of the leaves and pitchers were still located on the ground or nearly. We used the common allometric relation (Niklas, 2006): Leaf area = a* Leaf length^b on a sample of 20 leaves per species (10 from self-supporting individuals and 10 from climbing individuals). Allometries obtained are illustrated in Figure 1 and have been used for further estimation of leaf area (R Development Core Team, 2009).

A total of 173 individuals were described node by node from the apex to the last unequivocally measurable internode length. We chose to only describe the main axis. We described self-supporting, transitional and climbing individuals. Transitional plants were defined as plants that started to produce upper or transitional pitcher on its axis whose apical internode length dramatically increase. Plants were defined as lianescent if they are totally supported by vegetation and produce upper pitchers. *N. gracilis* was only described at this lianescent stage since it is a shrub whose stages are less clear and do not produce real self-supporting or transitional plants. Number of individuals described for each stage and species are given in Table 1. For each node we recorded: the internode length, the leaf length, the presence of a pitcher and its stage (developing, functional, dead, aborted), the pitcher length and, if present, the length of the waxy layer and the presence of an inflorescence, functional of senescent. Pitchers were measured from the upper part of the peristome (at the junction with the lid basis) to the lower part of the digestive zone. An R script was developed to convert individual descriptions into a graphical visualization that summarizes information described above (Figure 2).

To compare the activity of carnivorous and photosynthetic compartments between species and stages, we first extracted, for each individual, the number of active pitchers, the cumulated pitcher length, the proportion of aborted pitchers and the cumulated photosynthetic area (Figure 3). The ratio [waxy layer / total pitcher length], already shown to reveal ontogenic changes (Gaume & Di Giusto, 2009b) obtained are illustrated in Figure 4.

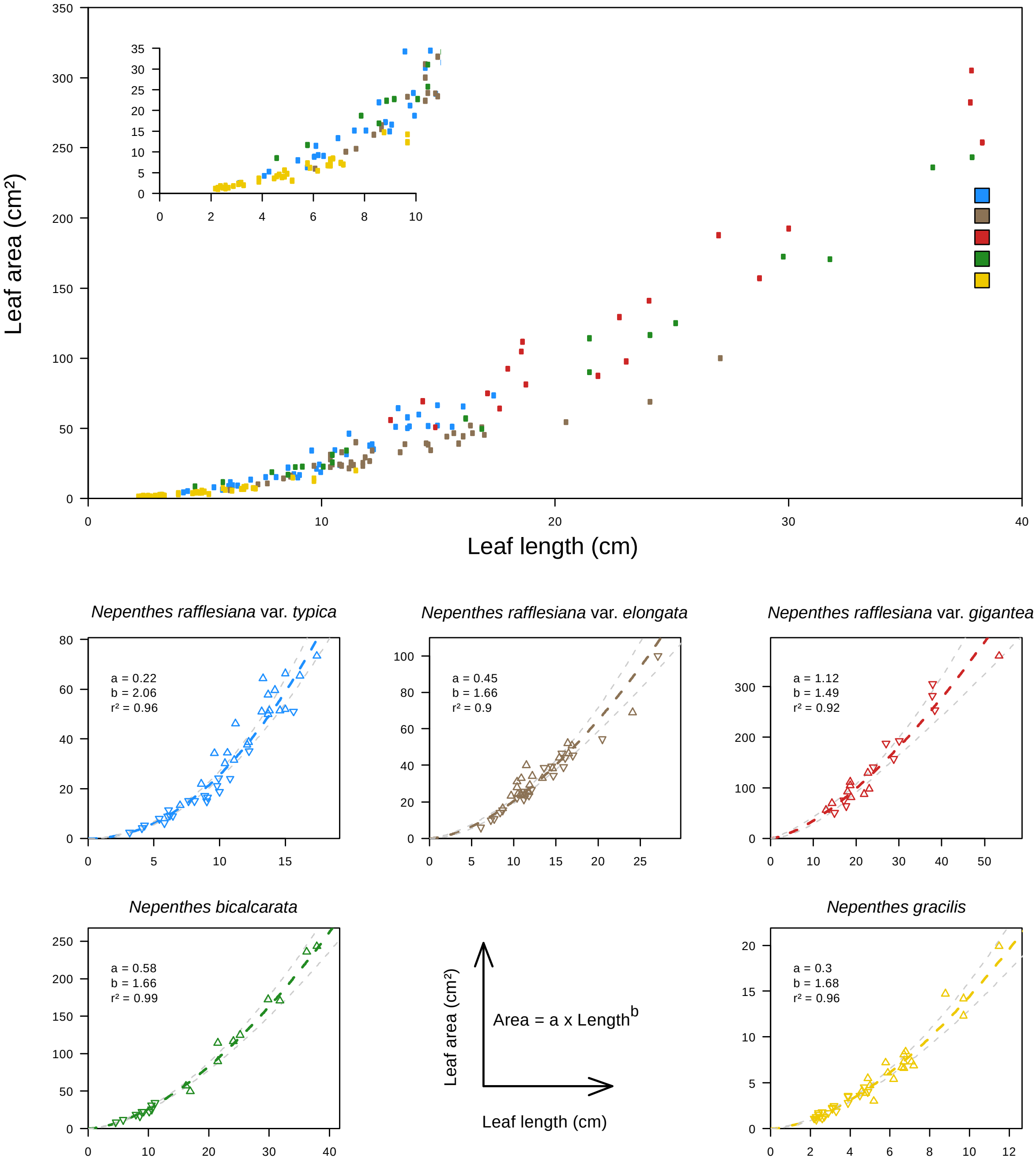

Figure 1: Leaf allometries obtained. Allometric coefficients are given for each species, and r^2 indicate the goodness of area estimation. Using these allometries, we were able to estimate the photosynthetic area whereas we simply measured each leaf length.

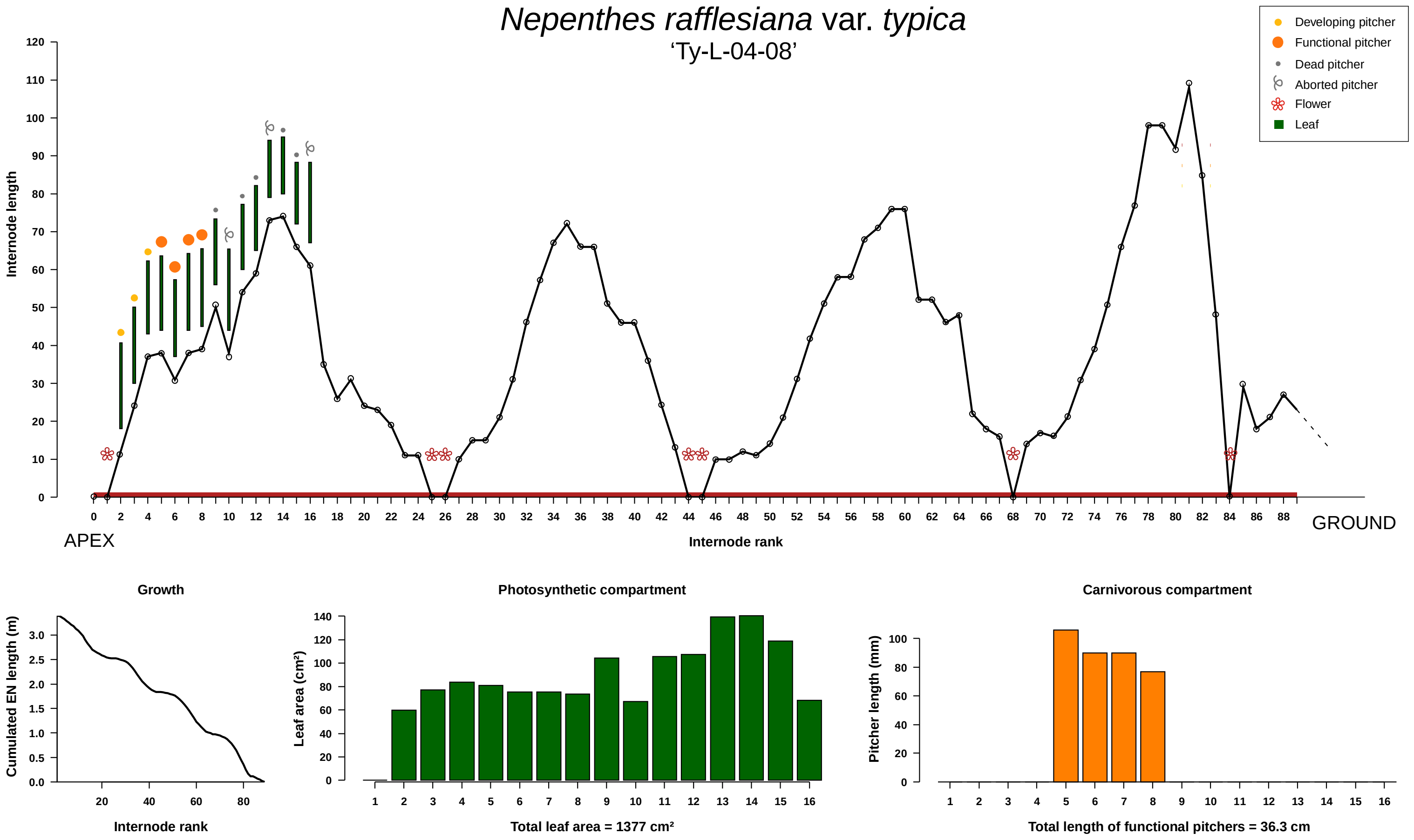

Figure 2: An example of the graphical display obtained by our R-script, here a climbing individual of *Nepenthes rafflesiana*. The upper figure illustrates the internode length (y-axis) measured node by node (x-axis) along the axis from the apex (left) to the ground (right). . From left to right, Figures on the bottom illustrate : i) cumulated internode length along the internode rank, ie the length of the main axis throughout time, ii) the distribution of estimated leaf areas and the total photosynthetic area (here 0,13 m^2) and iii) the distribution of the length of functional pitchers and the corresponding cumulated sum (here 0,36 m). The internode sequence illustrates that the growth is maximal between two annual flowering events. Some aborted pitchers are observed on this individual. The older leaves bear no longer functional pitchers whereas the younger leaves bear developing ones. Relatively few pitchers are functional at the same moment

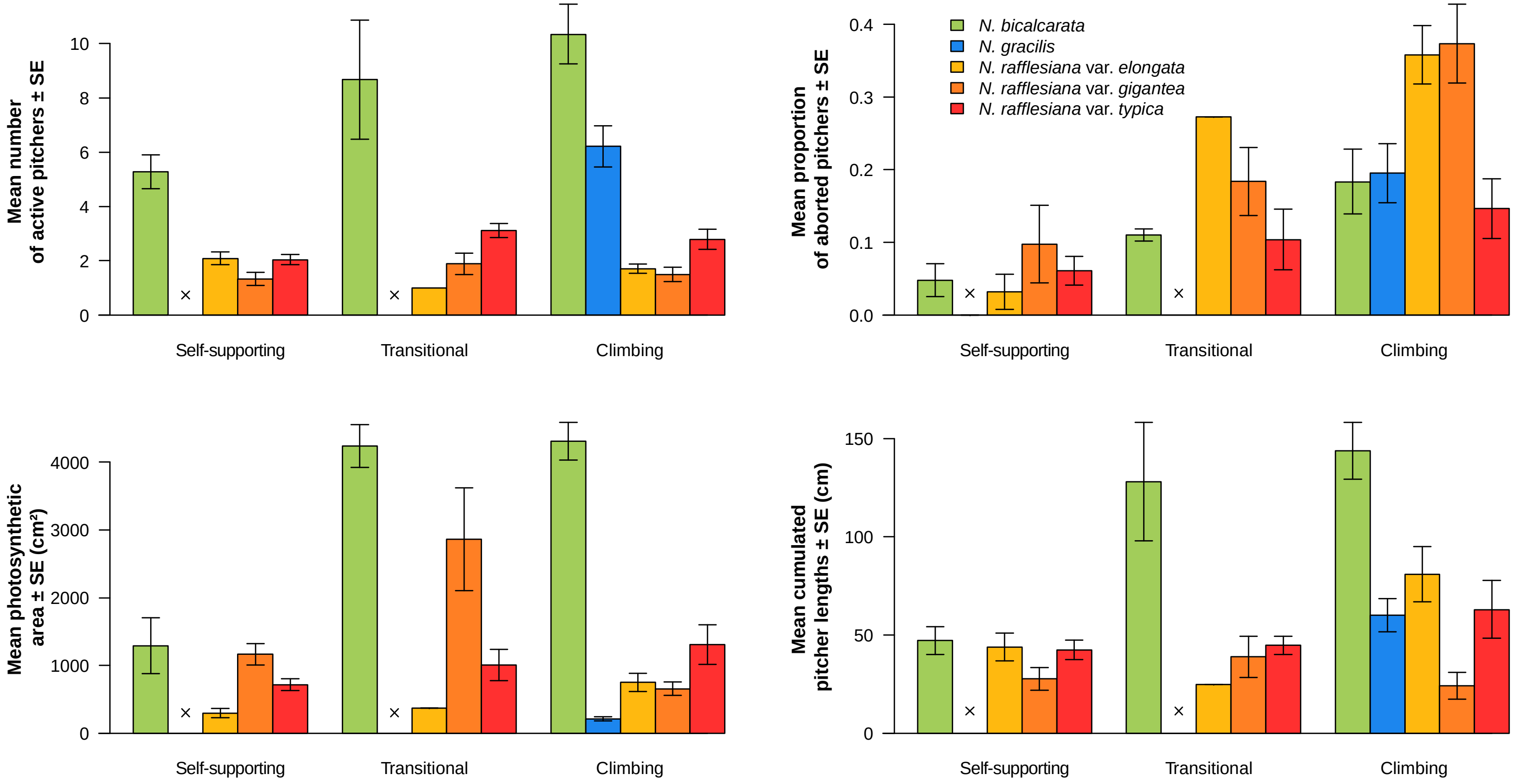

Figure 3: Barplots of four photosynthetic and carnivorous indexes for the five species studied, and for three ontogenic stages. *Nepenthes gracilis* do no produce true self-supporting or transitional stages. The proportion of aborted pitchers is higher in climbing stage than in self-supporting stage, which may reflects a balance between producing heavy traps and assuring a solid hanging in the surrounding vegetation. Among the species studied, *N. bicalcarata* possess the highest number of functional pitchers (which may be seen as an adaptation to its symbiotic ant) and the largest photosynthetic area. *N. gracilis* produces a lot of small pitchers, but the cumulated pitcher length (which could be a good proxy for carnivorous benefits) is comparable to those of other species. Besides these observations, there is no clear difference between species or between ontogenic stages. Further analyses including growth rates and pitcher lifespan will probably contrast these results.

Ontogenic stage	**Species**	**Number of functional pitchers**	$\mathbf{N_{ind}}$	$\mathbf{N_{pit}}$	**Photosynthetic area (cm²)**	$\mathbf{N_{ind}}$	$\mathbf{N_{pit}}$	**Proportion of aborted pitchers**	$\mathbf{N_{ind}}$	$\mathbf{N_{pit}}$	**Total pitcher length (cm)**	$\mathbf{N_{ind}}$	$\mathbf{N_{pit}}$
Self-supporting stage	*N. bicalcarata*	5.27 ± 2.05	11	58	1290 ± 1370	11	79	0.0482 ± 0.0755	11	78	47.1 ± 23.8	11	58
	N. rafflesiana var. *elongata*	2.1 ± 1.09	21	44	295 ± 303	21	164	0.0322 ± 0.11	21	133	43.8 ± 32.3	21	44
	N. rafflesiana var. *gigantea*	1.33 ± 0.707	9	12	1170 ± 518	11	91	0.0977 ± 0.177	11	91	27.6 ± 16.3	9	12
	N. rafflesiana var. *typica*	2.05 ± 0.844	22	45	713 ± 454	28	234	0.0613 ± 0.105	28	204	42.3 ± 26.1	22	45
Transitional stage	*N. bicalcarata*	8.67 ± 3.79	3	26	4240 ± 547	3	47	0.11 ± 0.0149	3	46	128 ± 52	3	26
	N. rafflesiana var. *elongata*	1	1	1	370 ± 0	1	11	0.273 ± 0	1	11	24.8 ± 0	1	1
	N. rafflesiana var. *gigantea*	1.89 ± 1.17	9	17	2870 ± 2400	10	133	0.184 ± 0.147	10	132	38.8 ± 31.4	9	17
	N. rafflesiana var. *typica*	3.11 ± 0.782	9	28	1010 ± 695	9	134	0.104 ± 0.125	9	130	44.7 ± 14.2	9	28
Climbing stage	*N. bicalcarata*	10.3 ± 3.8	12	124	4310 ± 962	12	222	0.184 ± 0.155	12	200	144 ± 49.9	12	124
	N. gracilis	6.21 ± 3.31	19	118	209 ± 151	20	319	0.195 ± 0.183	20	277	60 ± 38.1	20	126
	N. rafflesiana var. *elongata*	1.71 ± 0.686	17	29	751 ± 593	19	227	0.358 ± 0.175	19	222	80.9 ± 57.7	17	29
	N. rafflesiana var. *gigantea*	1.5 ± 0.756	8	12	656 ± 293	9	97	0.373 ± 0.162	9	94	24 ± 18.3	8	12
	N. rafflesiana var. *typica*	2.79 ± 1.62	19	53	1310 ± 1260	19	290	0.147 ± 0.179	19	272	62.9 ± 62.5	19	53
Total number			160	567		173	2048		173	1890		161	575

Table 1: Summary of the four photosynthetic and carnivorous indexes (as illustrated in Figure 3).

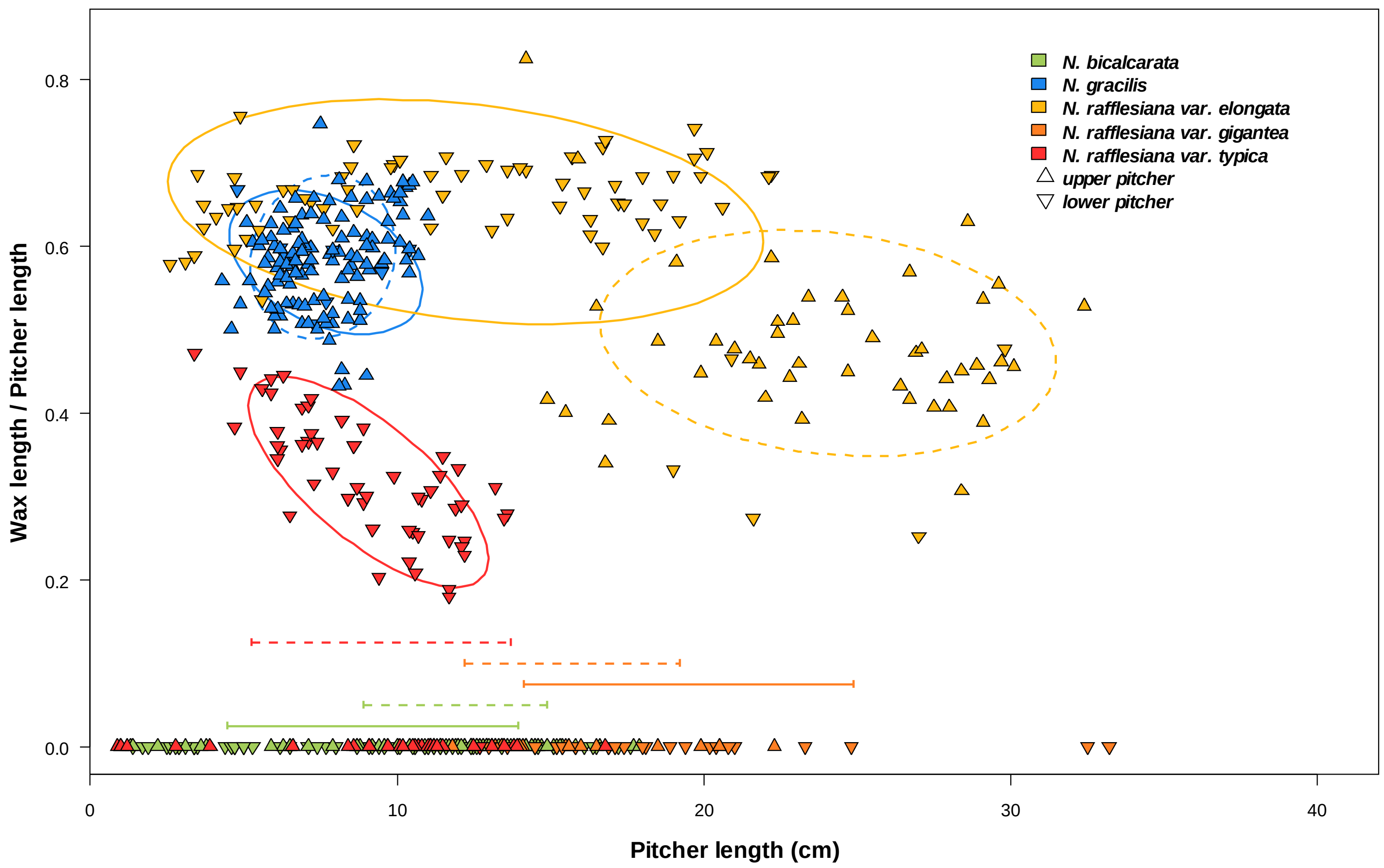

Figure 4: Pitcher lengths and wax proportion for lower and upper pitchers of the five studied species. Confidence (or segments) ellipses correspond to mean ± 1 SD; dashed lines correspond to upper pitcher, solid lines to lower pitchers. The pitchers of *Nepenthes bicalcarata* and *N. rafflesiana* var. *gigantea* never bear a waxy layer whereas those of the var. *typica* lost it throughout ontogeny and those of the var. *elongata* always present a waxy layer (see Di Giusto and Gaume, 2009). The pitchers of *N. gracilis* also always bear a waxy layer and the length of lower and upper pitcher is almost identical.

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ANNEXE 4 :TRAP PHYSIOLOGY AND ATTRACTIVENESS OF SIX LOWLAND *NEPENTHES* SPECIES

Vincent Bonhomme, Isabelle Gounand, Emmanuelle Jousselin and Laurence Gaume

During our 2009 field trip we conducted a survey of the trap physiology and attractiveness, i.e. the number of visitors, of six lowland species. All the data collected have not been analyzed yet but we present here the very first results, related to the aims of this thesis and which concern the dynamics of pH and viscoelasticity of the digestive liquid of these species, during the first month after the lid opening.

Aims of this study

We know that the pitchers of genus *Nepenthes* presents a functional diversity between species and for some species, between the dimorphic pitchers produced throughout ontogeny (Gaume & Di Giusto, 2009, Bonhomme *et al.*, in prep.-c). Physiological studies have focused so far on the mechanisms of nutrients absorption in strictly carnivorous species (Tökés *et al.*, 1974, Adamec, 1997, An *et al.*, 2001, An *et al.*, 2002a, An *et al.*, 2002b, Steinhauser *et al.*, 2006, Hatano & Hamada, 2008) and/or to bring evidence that they partly rely on others sources of nutrients (Moran *et al.*, 2001, Moran *et al.*, 2003, Clarke *et al.*, 2009). The comparative physiological studies available resulted from random measurements of functional pitchers of different age (Clarke, 1997) or focused on the instantaneous rates of nutrient absorption but not on the dynamics of pH through pitcher lifespan (Moran *et al.*, 2010). We nevertheless know that the acidity of the digestive liquid of *Nepenthes rafflesiana* var. *typica* reaches its maximum one week after the lid opening (pH ~2) while its viscoelasticity (observed without a ultra rapid camera) is maximal when the lid opens (Bauer *et al.*, 2008). Concerning the production of odors and its effect on insect attraction we know that the pitchers of *N. rafflesiana* var. *typica* produce floral scents (Di Giusto *et al.*, 2010) whose production increase when the pitchers age (Bauer *et al.*, 2009) but we do not have data as concerns others species.

This study will attempt to link i) the physiochemical characteristics of the pitcher: pH, viscoelasticity and volume of the digestive liquid, and ii) the attractiveness of the pitcher: odors produced, number and type of visitors and fill find some gaps in our comprehension of the functioning of *Nepenthes* pitchers, particularly:

- Is the pattern of a decreasing viscoelasticity when the pitchers age a general characteristics of viscoelastic species? Are the species described as never viscoelastic (e.g. *N. ampullaria*, *N. bicalcarata*, *N. gracilis* but see (Bonhomme *et al.*, in prep.-b)) are really never viscoelastic even at the very first days of opening?
- Similarly for the pH of the digestive liquid, do all species increase the acidity of their digestive liquid during the first week after the opening of the lid? Among the species studied, can we establish a link between a peculiar trapping strategy and digestion process and the acidity of the liquid? For instance, *N. bicalcarata* likely used its mutualistic ant to predigest prey and we know that it does not have a very acidic liquid, but does it present an acidification of the liquid during the first days that follows the opening of the lid? Since *N. ampullaria* is partly detritivorous, does it need such an acidic fluid? Is the volume of the digestive fluid adjusted by the plant?
- Do all species produce odors? Are the production of scents and insect attraction correlated with the maximal acidity and/or viscoelasticity of the digestive liquid fluids? Since the trapping strategies are associated with peculiar prey spectra (non-viscoelastic species mainly trap ants while viscoelastic species trap a richer prey spectrum including a significant proportion of flying insects), do the visitors attracted reflects the general trends of these prey spectra (Di Giusto *et al.*, 2008, Alaux, 2010). In other terms, is the attraction specialized towards peculiar insect types?

Methods and first results

10 lower and 10 upper pitchers of *N. ampullaria*, *N. bicalcarata*, *N. gracilis*, *N. rafflesiana* var. *elongata*, *N. rafflesiana* var. *typica*, *N. rafflesiana* var. *gigantea* were chosen and marked on different individuals ~5 days before their opening. The pitchers were then monitored for 35 days (ideally days 1, 3, 5, 7, 10, 15, 21, 28, 35 after lid opening).

We systematically observed the visitors of the pitchers for 2 min and noted their order before measuring the pH, the height of the liquid, performing qualitative assessments of pitcher odour. We sampled 100 µL of digestive liquid of each pitcher, stored in a 1.5 mL Eppendorf tube before viscoelasticity measurements performed when back at home following the protocol described in (Gaume & Forterre, 2007, Bonhomme *et al.*, in prep.-c).

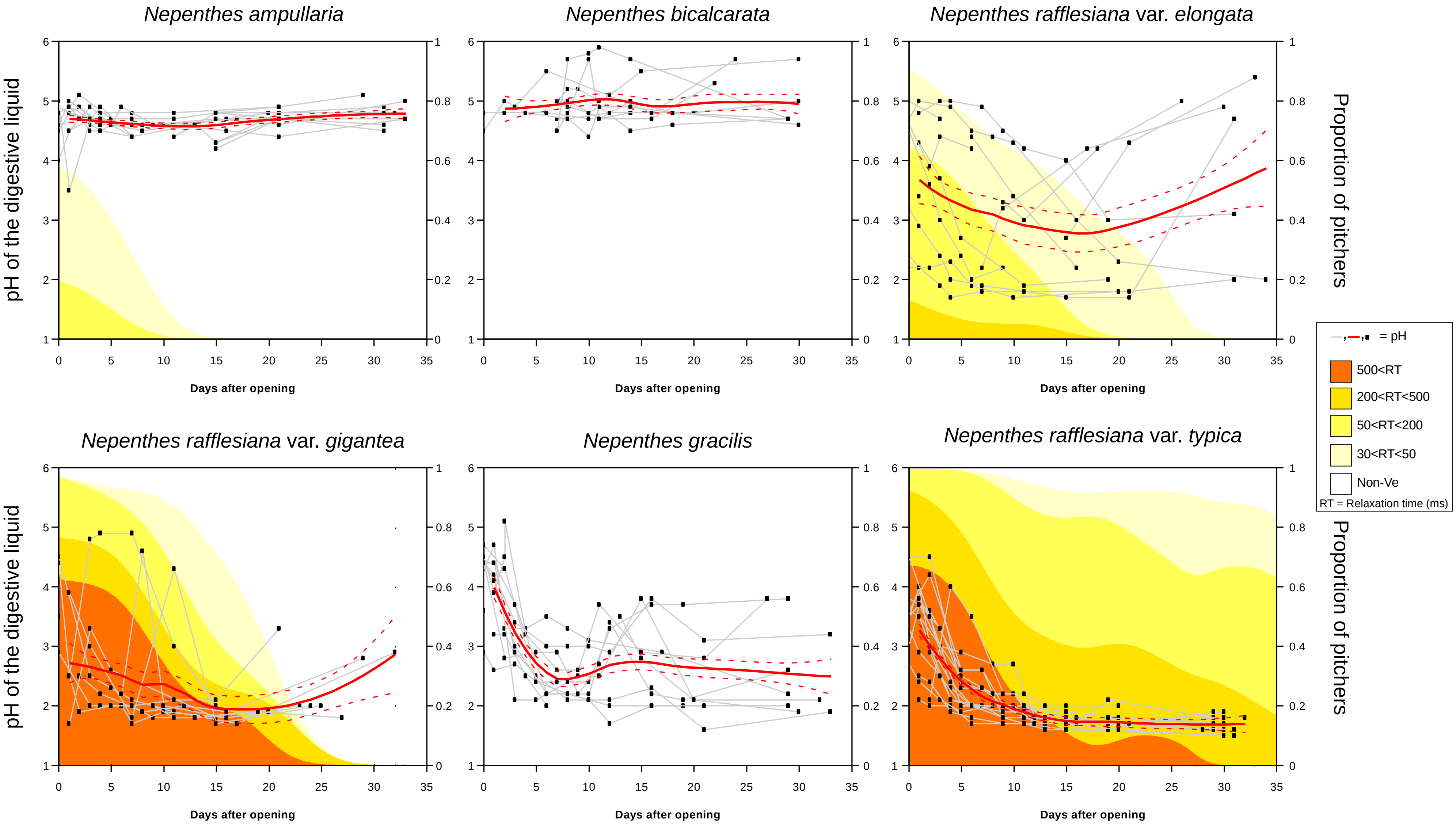
Nepenthes ampullaria
Nepenthes bicalcarata
Nepenthes rafflesiana var. elongata
Nepenthes rafflesiana var. gigantea
Nepenthes gracilis
Nepenthes rafflesiana var. typica
pH of the digestive liquid
Proportion of pitchers
Days after opening
0
5
10
15
20
25
30
35
1
2
3
4
5
6
0
0.2
0.4
0.6
0.8
1
= pH
500<RT
200<RT<500
50<RT<200
30<RT<50
Non-Ve
RT = Relaxation time (ms)

Figure 1 (preceding page): Dynamics of the pH and viscoelasticity of the digestive fluids of 6 lowland *Nepenthes*. Black dots are single measurements, grey lines link up measurements on the same individual, red lines is a polynomial regression with local fit (solid line) and confidence interval (dashed lines). Below pH data are plotted proportions of individuals with a given viscoelasticity value, from white (non-viscoelastic fluid) to orange (highly viscoelastic fluid as estimated by the relaxation times measured which are proportional to viscoelasticity).

As concerns pH two categories appear: *Nepenthes ampullaria* and *N. bicalcarata* have a weakly acidic fluid which acidity does not vary when pitchers age, contrary to all other species that present a marked diminution of their pH. For *N. rafflesiana* var. *elongata*, and in a lesser extent for *N. rafflesiana* var. *gigantea* extent the pH increases at the end of the survey which probably corresponds to the senescence of these pitchers. A similar profile would probably have been observed for *N. rafflesiana* var. *typica* and *N. gracilis* if we could have realized a longer monitoring. Concerning the dynamics of viscoelasticity, the most striking result is that the fluid of *N. ampullaria* is (weakly) viscoelastic during the first ten days after the opening of the lid for half of the pitchers surveyed. The fluids of *N. bicalcarata* and *N. gracilis* are never observed to be viscoelastic. For the varieties of *Nepenthes rafflesiana*, the dynamics of viscoelasticity seem to be correlated with the pH dynamics, and probably to the specific pitcher lifespan.

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Montpellier, 3 janvier 2011

Abstract

The carnivorous pitcher-plant genus *Nepenthes* encompasses at least 120 species that are distributed across a wide variety of habitats, mainly in the islands of SE Asia. The traps of these climbing plants are modified leaves that form pitchers and exhibit great morphological diversity . For years, people have believed that Nepenthes trapping systems solely rely on the properties of their slippery waxy layer, responsible for the fall of insects into the pitcher. Yet in *Nepenthes rafflesiana*, this waxy layer is lost during ontogeny, resulting in a change in the pitcher shape. The digestive fluid of this species has been found to have viscoelastic properties, that appear to play a key role in retaining prey in the trap. Is this waxy layer a widespread character in the genus? Is it an ancestral character lost in some species, favoring the emergence of new forms of trap? Have these changes appeared several times independently, in response to similar ecological selection pressures? Similarly, is the viscoelastic character widespread in the genus or is it only present in non-waxy species?

This thesis explores the morphological and functional diversity of the genus *Nepenthes*, and the selection pressures and developmental mechanisms that may have led to this diversity. First, experiments on several species show that they differ in their capture success, and that there is a trade-off between amounts of wax produced in the pitchers and the degree of viscoelasticity of the digestive fluid. Lowland species are more often associated with aqueous fluids, whereas mountain species are associated with viscoelastic fluids; in addition, the most viscoelastic species do not exhibit a waxy layer. Second, we show that the capture efficiency of wax and the viscoelastic fluid differs depending on the type of prey (ants or flies); the wax is only effective on ants, whereas the viscoelastic fluid is lethal for both types of insect. Therefore, the entomofauna endemic to the habitats of lowland or mountain species are likely to exert a selection pressure favoring a particular trapping strategy. Finally, we show that in a peat forest of Brunei (Borneo), a *Nepenthes* species lacking both the waxy layer and viscoelastic fluid captures its prey via a mutualist, which hunts by ambushing prey into the pitcher.

A molecular phylogeny based on AFLPs, four chloroplastic markers and ITS, improves the phylogenies available and has allowed us to reconstruct the evolutionary history and origin of these characters. We show that the viscoelastic trait is widespread, it is probably plesiomorphic and might be inherited from a common ancestor with the sister genera *Drosera* and *Drosophyllum*; these genera are also carnivorous, but exhibit a very different trapping system. Ontogenetic loss of the waxy layer is a derived character which has appeared several times independently, and is connected with the appearance of funnel-shaped pitchers that possess a viscoelastic liquid. We propose an evo-devo mechanism that unifies all ontogenetic patterns observed so far and illustrates how heterochronies, such as the ontogenetic loss of the waxy zone, can be an important mechanism for the morphological and functional diversification in these carnivorous plants.

Keywords: *Nepenthes*, carnivorous plant, phylogeny, heterochronies, morphological diversity, trapping strategies, plant-insect interactions, epicuticular wax, viscoelastic fluid, Borneo.

Résumé

Le genre de plante carnivore à urnes *Nepenthes* comprend au moins 120 espèces, réparties principalement dans les îles du sud-est asiatique, dans une grande variété d'habitats. Les pièges de ces espèces lianescentes sont des feuilles modifiées en urnes qui présentent une grande diversité morphologique. Pendant longtemps, on a cru que leur système de capture reposait uniquement sur les propriétés glissantes de leur couche cireuse responsable de la chute des insectes. Pourtant chez *Nepenthes rafflesiana* au moins, cette zone cireuse est perdue au cours de l'ontogénie, induisant un changement de forme du piège. Le fluide digestif de cette espèce s'est révélé avoir des propriétés viscoélastiques déterminante pour la rétention des proies dans ses pièges. Est-ce que la plupart des espèces ont une zone cireuse ? Ce caractère est-il ancestral et perdu au cours de l'évolution chez certaines espèces favorisant l'apparition de nouvelles formes de piège? Est-ce que ces changements sont apparus plusieurs fois indépendamment en réponse à des pressions de sélection écologiques similaires ? Qu'en est-il du fluide digestif ? Le caractère viscoélastique est-il répandu ou ne concerne-t-il que les espèces dépourvues de cire ?

Cette thèse explore la diversité fonctionnelle et morphologique au sein du genre *Nepenthes* ainsi que les pressions de sélection et les mécanismes développementaux qui ont pu être à son origine. Nous montrons d'abord au travers d'expériences menées sur plusieurs espèces que celles-ci diffèrent dans leur succès de capture, et dans les quantités de cire et le degré de viscoélasticité de leur fluide et sont soumises à des *trade-off*. Les espèces de plaines sont plus souvent associées à des fluides aqueux alors que les espèces de montagne sont plutôt associées à des fluides viscoélastiques, les plus élastiques étant toujours dépourvues de cire. Nous montrons ensuite que l'efficacité de capture de la cire et du fluide diffère selon le type de proies (fourmis ou mouches) considérées, la cire étant efficace seulement sur les fourmis, le fluide viscoélastique étant létal pour les deux types d'insecte. L'entomofaune caractérisant les habitats de plaine ou de montagne exerce donc vraisemblablement une pression de sélection pour une stratégie de piégeage donnée. Nous montrons par ailleurs comment dans les forêts tourbeuses du Brunei (Bornéo) un *Nepenthes* dépourvue de cire et de liquide viscoélastique capture ses proies à l'aide d'une fourmi mutualiste qui chasse en embuscade dans ses urnes.

L'obtention d'une phylogénie moléculaire, basée sur des AFLP, 4 marqueurs chloroplastiques et l'ITS, améliore les phylogénies disponibles et permet de reconstituer l'histoire évolutive et l'origine de ces caractères. Nous montrons que le caractère viscoélastique est très répandu, vraisemblablement plésiomorphe et hérité d'un ancêtre commun aux genres frères *Drosera* et *Drosophyllum*, également carnivores mais aux pièges morphologiquement différents. La perte ontogénétique de la zone cireuse est un caractère dérivé, apparu plusieurs fois indépendamment et relié à l'apparition d'urnes en forme d'entonnoir et au liquide viscoélastique. Nous proposons un mécanisme évo-dévo qui unifie tous les patrons ontogénétiques observés et qui illustre comment des phénomènes hétérochroniques, telle que la délétion ontogénétique plus ou moins rapide de la zone cireuse, peuvent être source de diversification morphologique et fonctionnelle chez ces plantes carnivores.

Mots-clés: *Nepenthes*, plante carnivore, phylogénie, hétérochronies, diversité morphologique, systèmes de piégeage, interactions plante-insectes, cire épicuticulaire, fluide viscoélastique, Bornéo.