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A STICKY SITUATION: ASSESSING ADAPTATIONS FOR PLANT CARNIVORY IN THE CARYOPHYLLALES BY MEANS OF STOCHASTIC CHARACTER MAPPING

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Phylogenetic relationships among carnivorous plants of the angiosperm order Caryophyllales have been explored, although a robust phylogeny encompassing all carnivorous genera is absent. We sample nuclear ribosomal spacer (internal transcribed spacer) and chloroplast intergenic spacer (PY-IGS), along with previously sequenced DNA from members of the noncore Caryophyllales, for use in Bayesian statistics and maximum likelihood–based searches of phylogeny. Taxonomic relationships across genera are refined, and three strongly supported clades are identified: monophyletic Droseraceae, Nepenthaceae, and a third clade containing Ancistrocladaceae, Dioncophyllaceae, and Drosophyllaceae. In combination with phylogenetic reconstruction, stochastic character mapping is used to assess evolutionary changes in the morphology of glands that are found on the lamina and involved in the digestion of prey. The presence of sessile glands is identified as the likely ancestral character state, and stalked and pitted glands are suggested to have been acquired independently by ingroup and outgroup taxa. Additionally, in some genera we found a lack of association between gland vasculature and plant carnivory, demonstrating that the internal architecture of glands is not indicative of whether the plant is a functional carnivore. Finally, we discuss how adaptive changes resulting in the evolution of the carnivorous gland may have occurred either by emargination of the leaf blade or homologous transformation of pinnae.

Keywords: carnivorous plants, Caryophyllales, chloroplast intergenic spacer (PY-IGS), digestive glands, internal transcribed spacer, phylogeny.

Introduction

Phylogenetic Relationships among the Carnivorous Genera of the Caryophyllales

Léon Croizat once proposed that all carnivorous plants comprised a single lineage, on the basis of similarities in trap type, and that carnivory represented an early condition of the angiosperms (Croizat 1960). More recently, however, carnivory has been shown to be a derived condition and is hypothesized to have arisen independently at least five times within angiosperms—in the angiosperm orders Ericales, Lamiales, Oxalidales, Poales, and Caryophyllales (APG II 2003)—suggesting that convergent evolution of the carnivorous habit occurred across angiosperms. The greatest number of carnivorous plant species are found in the noncore Caryophyllales (Guénoud et al. 2002), in the families Droseraceae (Aldrovanda, Dionaea, and Drosera), Drosophyllaceae (Drosophyllum), Nepenthaceae (Nepenthes), and Dioncophyllaceae (Triphyophyllum; Rice 2006). The carnivorous plant lineage of the Caryophyllales is also unique in that it appears as though plant carnivory arose once and was subsequently lost by closely related members of Ancistrocladaceae (Ancistrocladus) and Dioncophyllaceae (Dioncophyllum and Habropetalum; Heubl et al. 2006).

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Previous phylogenetic analysis of the carnivorous Caryophyllales have focused sampling on species within Droseraceae (Rivadavia et al. 2003), Nepenthaceae, and Ancistrocladaceae (Meeberg et al. 2000, 2006; Heubl et al. 2006; Meimberg and Heubl 2006) independently or have limited sampling designed to test the placement of Dionaea and Aldrovanda (Cameron et al. 2002). Gene regions used in these analyses tend to be either slowly evolving markers (rbcL, matK, and atpB) selected to investigate backbone relationships or more rapidly evolving markers (18S, trnK, and peptide transferase 1 [PTR1]) selected to investigate species-level relationships within a given family or genus. In this study, we added the chloroplast intergenic spacer between psaA and ycf3 (PY-IGS) and nuclear ribosomal DNA (internal transcribed spacer [ITS]) to previously published molecular data (atpB, matK, petB, PTR1, rbcL, and trnK) for analyses of phylogenetic relationships among the carnivorous plants of the Caryophyllales. We sample all carnivorous genera, including multiple representatives of each genus where possible, to test phylogenetic relationships among genera.

Glands Involved in Plant Carnivory in the Caryophyllales

The noncore Caryophyllales share a number of synapomorphies, including the possession of pitted, sessile, and stalked glands (Judd et al. 2002). In Plumbaginaceae and Polygonaceae, families sister to the carnivorous plants of the Caryophyllales, glands are rarely vascularized and function in the secretion of salt in halophytic conditions, in the secre-
tion of mucilage to deter herbivory, or for dispersal (i.e., epizoochory; Wilson 1890; Lütge 1971; Fahn and Werker 1972; Sakai 1974; Faraday and Thomson 1986). In carnivorous genera, homologous glands have apparently evolved to function in the secretion of digestive enzymes and to absorb amino acids and other organic nutrients (Morrissey 1964; Amagase 1972; Juniper et al. 1989; Owen et al. 1999; Eilenberg et al. 2006; Hatano and Hamada 2008). Of the three gland types that function in plant carnivory, sessile and stalked glands have diversified extensively to include vascularized forms.

Members of Droseraceae include the sundews (genus Drosera), in addition to the monotypic Venus flytrap (Dionaea muscipula) and its aquatic sister species Aldrovanda vesiculosa (Cameron et al. 2002; Rivadavia et al. 2003). Species of Droseraceae can have either sessile glands or a combination of sessile and stalked glands. In Drosera, two basic types of carnivorous glands are present: (1) vascularized stalked glands and (2) nonvascularized sessile glands. The upper leaf surface of Drosera is densely covered with both types of gland, whereas only type 2 is found on the abaxial side of the leaf, petioles, and inflorescence scapes (Juniper et al. 1989). Cells of type 1 are either epidermal or parenchymatous in origin with trichaeids and xylem embedded within, which extend into the veins of the leaves (Gilchrist and Juniper 1974). Conversely, the cells of type 2 are exclusively epidermal in origin and lack vasculature at maturity (Juniper et al. 1989). In D. muscipula, nonvascularized sessile glands are located abaxially or adaxially on the plant trap. Glands found on the abaxial side of the leaf are stellate, whereas glands on the adaxial side (those involved in digestion) are made up of a variety of cell types, which include a single basal cell, a stalk cell, and eight or more digestive gland cells (Scala et al. 1968). In addition to the sessile glands on the digestive surface of the trap, trigger hairs can be found adaxially and centrally on each lobe of the lamina (Juniper et al. 1989). Interlocking vascularized “teeth” are present at the leaf margin and are homologous in their position to the marginally located stalked glands in Drosera (Juniper et al. 1989). In the sister species A. vesiculosa, nonvascularized sessile glands are present in each trap and may be either four-lobed when placed adaxially or two-lobed when placed abaxially (Ashida 1935). Similarly to D. muscipula traps, A. vesiculosa traps have teeth at the margin of the leaf.

The genus Drosophyllum contains the single species D. lusitanicum, a carnivorous plant with a shrublike habit that lives among dry, alkaline soils (Harshberger 1925). Attached to the leaves of D. lusitanicum are both stalked and sessile glands accompanied by a network of vascular bundles (Juniper et al. 1989). Drosophyllum is currently placed in its own family, Drosophyllaceae, with reported affinities to the Dioncophyllaceae (see “Discussion”). Triphyophyllum peltatum (Dioncophyllaceae), a plant considered to be carnivorous during some periods of its development, has sessile and stalked glands similar in architecture to those of D. lusitanicum—being filled with a central vessel of xylem and phloem (Green et al. 1979). Interestingly, Dioncophyllum tholloni, a close sister taxon to T. peltatum (fig. 1), is not considered carnivorous and does not display glands on its lamina.

Closely related to Dioncophyllaceae is the family Ancistrocladaceae, a group of noncarnivorous lianas from Africa and Asia (Foster and Sork 1997; Gereau 1997; Cheek 2000; Taylor et al. 2005). In members of Ancistrocladaceae, glandular pits that function in wax secretion can be found on the abaxial side of the lamina (Taylor et al. 2005). Although not vascularized, Ancistrocladaceae glands are approached by vasculature that terminates abruptly below the pitted glands (Metcalfe 1951).

In Nepenthes (Nepenthaceae), a genus of ~130 species native to tropical Asia (McPherson 2010), pitted glands partially covered by the epidermis are located at the base of a modified lamina (the pitcher), which acts as the carnivorous trap. Gland structure is defined by one or more layers of secretory and endodermal cells overlaid with columnar cells and a cuticle (Owen et al. 1999). Tracheids are often found near these endodermal cells, although the glands themselves do not contain xylem and phloem (Lloyd 1942; Rottloff et al. 2009).

Although the glands present in the carnivorous Caryophyllales have been described previously in great detail, assessments of gland homology have not resulted in a unanimous conclusion as to their developmental origin. In more recent articles and reviews, digestive glands have been considered homologous with trichomes (Heubl et al. 2006), hairs (Chase et al. 2009), or epidermal cells (Owen and Lennon 1999). Defining the carnivorous gland may be confounded by differences in gland morphology between genera. To investigate the evolution of gland morphology, we explore the morphology of glands among carnivorous genera by means of stochastic character mapping to further understand how carnivorous glands may have evolved in the Caryophyllales.

The aims of this study are to (1) evaluate past phylogenetic analyses that included carnivorous taxa of the Caryophyllales, (2) determine the utility of the PY-IGS and ITS molecular markers for phylogenetic reconstruction at the level of genus and species in the Caryophyllales, and (3) investigate gland morphology among carnivorous taxa and closely related noncarnivorous taxa under a phylogenetic framework in order to identify characteristics that are key in determining whether glands found on the lamina of the leaf are indicative of plant carnivory.

Material and Methods

Taxon Sampling and Tissue Collection

A total of 51 taxa from the families Ancistrocladaceae, Dioncophyllaceae, Droseraceae, Drosophyllaceae, Plumbaginaceae, Nepenthaceae, and Polygonaceae were included in our analyses (appendix). Taxa were selected to represent biogeographic diversity across the carnivorous Caryophyllales. The samples collected originated mainly from living collections at the University of California Botanical Garden, the Missouri Botanical Garden, Universität Würzburg, and California Carnivores in Sebastopol, California. Freshly collected leaf tissue was preserved in silica gel and frozen at −80°C, and vouchers were deposited at the University of California herbarium. Dioncophyllum tholloni, a species not easily obtained from living collections, was collected in the wild by Gretchen Walters (MO) and vouchered, and tissue was sampled from the herbarium sheet. We were unable to obtain tissue for Habropetalum dawei (Dioncophyllaceae).
Fig. 1 Combined nuclear DNA, nuclear ribosomal DNA, and chloroplast DNA molecular marker phylogenetic reconstruction for the carnivorous Caryophyllales. Shown are results from maximum likelihood and Bayesian inference analyses of a concatenated internal transcribed spacer, chloroplast intergenic spacer (PY-IGS), atpB, petB, matK, PTR1, rbcl, and trnK data set for 19 taxa. Posterior probabilities and bootstrap support from complete analysis are indicated (bootstrap support value/posterior probability value) at nodes on the Bayesian 50% majority rule tree. Carnivorous taxa are indicated by an asterisk and are in bold typeface.
**Molecular Marker Sampling**

The nuclear ribosomal DNA including internal transcribed spacer (ITS) and the chloroplast intergenic spacer between psaA and ycf3 (PY-IGS) were chosen for phylogenetic reconstruction on the basis of previous studies demonstrating the utility of these markers for resolution at both the genus and species levels within the eudicots and Nepenthaceae (Downie et al. 1996; Sang et al. 1997; Shi et al. 2001; Meimberg 2002; Tan et al. 2002; Tate et al. 2003; Alejandro et al. 2008; Miranda et al. 2010). A previous study attempted to use ITS in Droseraceae but had unsatisfactory results (Miranda et al. 2010). Total genomic DNA was extracted from leaf base (Al-drovanda and Dionaea), leaf lamina (Ancistrocladus, Dioncophyllum, Drosera, and Drosophyllum), and the lamina-like region of the leaf base (Nepenthes and Triphyophyllum) by means of a cetyltrimethyl ammonium bromide (Doyle and Doyle 1987) or modified sodium dodecyl sulfate and sodium chloride protocol (Edwards et al. 1991). Extracted genomic DNA was quantified using a NanoDrop 1000 instrument (Thermo Fisher Scientific, Wilmington, DE).

Previously published sequences for atpB, matK, petB, PTR1, rbcL, and trnK were obtained from GenBank (http://www.ncbi.nlm.nih.gov/) for phylogenetic analyses (appendix). Of these molecular markers, only matK was available for H. daewi (GenBank: AF204845). To minimize missing data, outgroup genera Limonium (Plumbaginaceae) and Polygonum (Polygonaceae) were formed from composite sampling of atpB, ITS, matK, petB, PTR1, PY-IGS, rbcL, and trnK sequences from multiple species where necessary. Taxa used in our analyses are summarized in the appendix, along with voucher information and the GenBank accession number for each DNA sequence.

**PCR and DNA Sequencing**

Previously published primer pairs ITS5a (Downie et al. 1996) and ITS4 (White 1990) were used to amplify ITS in 10-µL aliquots with 10–100 ng of genomic DNA and the following reagents: 0.02 U of iProof polymerase (Bio-Rad, Hercules, CA), 1× HF iProof buffer (Bio-Rad), 2.0 mM MgCl₂, 0.2 mM of each dNTP, 0.5 µM of each primer, 0.025 mg/mL BSA, and 100% DMSO. PCRs were run on a MyCycler thermal cycler (Bio-Rad) under the following conditions: an initial denaturation at 98°C for 3 min; followed by 30 cycles of 98°C for 10 s, 54°C for 25 s, 72°C for 30 s (increasing 0.03°C with each cycle); and ending with a final extension at 72°C for 7 min. PG1f and PG2r (Tan et al. 2002) were used to amplify PY-IGS from the majority of species by means of the three-step PCR protocol with the annealing temperature set at 50°C.

Before sequencing, PCR products were purified using exonuclease I and shrimp alkaline phosphatase to remove single-stranded primers and remaining dNTPs (Fermentas International, Burlington, Ontario). PCR products were cycle sequenced using PCR primers and the ABI Prism BigDye Terminator Cycle Sequence Ready Reaction kit (ver. 3.1; Perkin-Elmer/Applied Biosystems, Foster City, CA). Products of cycle sequencing were resolved on an ABI Prism 3100 automated sequencer (Applied Biosystems).

**Alignment and Phylogenetic Analyses**

Forward and reverse sequences for ITS and PY-IGS were assembled and edited with Sequencer (ver. 4.7; Gene Codes, Ann Arbor, MI). A multiple sequence alignment for 19 taxa (appendix) was constructed from ITS, PY-IGS, atpB, matK, petB, PTR1, rbcL, and trnK with ClustalX under default settings (Thompson et al. 1994), with subsequent manual adjustment in Mesquite (ver. 2.72; Maddison and Maddison 2010). We excluded regions from ITS, PY-IGS, atpB, matK, petB, PTR1, rbcL, and trnK alignments that were poorly aligned across the entire data set and combined all sequences into a single concatenated data set (hereafter referred to as the “combined data set”) with a final length of 9988 bp.

Bayesian inference (BI) of phylogeny was conducted in MrBayes (ver. 3.1.2; Ronquist and Huelsenbeck 2003) using the combined data set and partitioned for each molecular marker under the best-fit model of evolution as determined by the Akaike Information Criterion (AIC) in jModelTest (ver. 0.1.1; Posada 2008): general time-reversible model (GTR; PTR1), GTR with γ correction (GTR+G; atpB, matK, petB, and trnK), or GTR with invariant sites and γ correction (GTR+I+G; ITS, PY-IGS, and rbcL). Two Bayesian analyses were performed simultaneously with posterior probabilities of the generated trees approximated using the Metropolis-coupled Markov chain Monte Carlo (MCMC) algorithm with four incrementally heated chains for 22,000 generations while sampling trees every 100 generations until both analyses converged on similar log-likelihood scores (the average standard deviation of split frequencies was <0.01). The first 110 trees were discarded as burn in, and a 50% majority rule tree was assembled from the remaining trees. Our Bayesian analyses including matK from H. daewi were unable to converge; this was most likely an artifact of increased missing data. Therefore, molecular data for H. daewi were removed from our phylogenetic analyses.

Maximum likelihood (ML) searches were performed using the interactive GARLI OSX GUI interface (Zwickl 2006) with an unpartitioned version of the combined data set for 1000 bootstrap replicates. A consensus of bootstrap trees was constructed with SumTrees (ver. 3.0.0) using the DendroPy Phylogenetic Computing Library (ver. 3.7.1; Sukumaran and Holder 2010). Trees were edited in Mesquite (ver. 2.72; Maddison and Maddison 2010) and Adobe Illustrator.

Two Bayesian analyses of a data set containing ITS and PY-IGS for 51 taxa (appendix) were performed to compare tree topology with the combined data set of eight nuclear DNA, nuclear ribosomal DNA, and chloroplast DNA molecular markers. These analyses were made simultaneously in MrBayes (ver. 3.1.2; Ronquist and Huelsenbeck 2003) with
GTR+G+I as determined by the AIC in jModelTest (ver. 0.1.1; Posada 2008). Posterior probabilities of the generated trees were approximated using the MCMC algorithm with methods similar to those for the combined eight-molecular-marker data set. The first 2500 trees were discarded as burn in, and a 50% majority rule tree was assembled from the remaining trees as unrooted.

**Character State Reconstructions**

Ancestral character reconstructions were conducted using maximum parsimony in MacClade (ver. 4.08 OSX; Maddison and Maddison 2005), ML in Mesquite (ver. 2.72; Maddison and Maddison 2010), and Bayesian stochastic character mapping (Huelsenbeck et al. 2003) in SIMMAP (ver. 1.5; Bolhback 2006). Character reconstruction results were consistent among these methods; therefore, we describe only the Bayesian approach here.

We used the 50% majority rule tree generated in our BI analyses for the combined data set to create stochastic mappings of gland types found on the lamina of the leaf and vasculature tissues associated with glands in SIMMAP. To assess evolutionary changes in gland morphology specifically related to plant carnivory, we scored character states as unordered for three gland types, taking into account the types of vasculature that can be found in each: for sessile glands, 0 = absence, 1 = presence, and 2 = xylem and phloem; for stalked glands, 0 = absence, 1 = presence, 2 = xylem, and 3 = xylem and phloem; and for pitted glands, 0 = absence and 1 = presence. Descriptions of genera and illustrations of micrographs were used to determine character states and a summary of basic gland types and their morphologies. We chose to set bias and rate parameters with priors determined by a MCMC configuration calculated in SIMMAP for each gland type. For sessile glands, the bias parameter was set to equal 1/k, and we used a gamma rate prior with shape parameters a = 1.062 and b = 0.049. For stalked glands, the bias parameter was similarly set to equal 1/k, and a gamma rate prior with shape parameters a = 0.671 and b = 0.011 was used. Last, for sessile glands a = 5.946 was set as the beta distribution prior for the bias parameter, and we used a gamma rate prior with shape parameters a = 0.972 and b = 0.019. Default values for the number of categories (k) for both beta and gamma distributions were used. Ancestral states at each node were calculated as the marginal posterior probability of each possible character state, which is dependent on the branch lengths and topology of the phylogenetic tree given (fig. 3).

**Results**

**Phylogenetic Reconstruction**

ML and BI methods of phylogenetic reconstruction gave congruent topologies that support monophyly of the carnivorous plant families of the Caryophyllales. Three strongly supported clades were identified corresponding with (a) a monophyletic Droseraceae, (b) a monophyletic Nepenthaceae, and (c) a third clade containing members of Ancistrocladaceae, Dioncophyllaceae, and Drosophyllaceae (figs. 1, 2).

In ML and BI reconstructions of phylogeny for the combined data set (fig. 1), Nepenthaceae (100 bootstrap support [BS] value/1.00 posterior probability [PP] value) is placed as sister to the clade containing members of Ancistrocladaceae, Dioncophyllaceae, and Drosophyllaceae with relatively strong support (89 BS/1.00 PP). The monotypic Drosophyllum is supported (100 BS/1.00 PP) as sister to a clade containing Dioncophyllaceae and Ancistrocladaceae. Within the Dioncophyllaceae, Dioncophyllum and Triphyophyllum are sister (1.00 BS/1.00 PP) and together are sister to a clade containing all included members of the genus Ancistrocladius (Ancistrocladaceae; 100 BS/1.00 PP) with high support (100 BS/1.00 PP). Droseraceae is recovered as monophyletic (97 BS/1.00 PP), with Dionaea and Aldrovanda sister to members of Drosera. Last, Dionaea and Aldrovanda are sister to one another with high support (100 BS/1.00 PP).

To determine whether tree topology for the carnivorous Caryophyllales is conserved among data sets, we compared ML and BI reconstructions for the combined data set (fig. 1) to a BI reconstruction for a data set including ITS and PY-IGS for a greater number of taxa (fig. 2). Topology was relatively consistent at the genus level between reconstructions with the exception of the position of Drosophyllum, which forms a polytomy with Dioncophyllum and Triphyophyllum with relatively high support (0.94 PP). It is also evident that the ITS and PY-IGS reconstruction does not fully resolve evolutionary relationships between taxa. This is especially true for Nepenthaceae, where ITS and PY-IGS are not phylogenetically informative enough to resolve relationships at the species level.

**Ancestral Reconstruction**

Stochastic character mapping revealed the presence of non-vascularized sessile glands alone (stalked and pitted glands absent) as the ancestral state for the carnivorous Caryophyllales (fig. 3A). Sessile glands were then lost in the lineage leading to Nepenthaceae and the clade containing Ancistrocladaceae, Dioncophyllaceae, and Drosophyllaceae (0.94 PP). Within this group, vascularized sessile glands containing both xylem and phloem arose secondarily and independently in Drosophyllum and Triphyophyllum. Within the taxa studied here, there are no known sessile glands associated only with xylem.

Stochastic character mapping also infers three independent origins of stalked glands within the ingroup (fig. 3B). Independently, the presence of stalked glands is an ancestral character state for outgroups (0.55 PP) and for Droseraceae (0.98 PP). The stalked glands of Droseraceae are vascularized with xylem, and according to our analyses these glands are secondarily lost in the flytrap lineages Dionaea and Aldrovanda (absence of stalked glands: 0.60 PP for Dionaea and Aldrovanda but 0.01 PP for Drosera). In Drosophyllum and Triphyophyllum, stalked glands vascularized with xylem and phloem are gained independently.

According to our reconstruction results, pitted glands are gained independently by ingroup and outgroup taxa (fig. 3C). Pitted glands are found in Limonium and Polygonum and are reconstructed as the ancestral condition of the outgroup. Pitted glands are also found in Nepenthaceae and...
Fig. 2  Internal transcribed spacer (ITS) and chloroplast intergenic spacer (PY-IGS) phylogenetic reconstruction for the carnivorous Caryophyllales. Shown are results from Bayesian inference analyses of ITS and PY-IGS for 51 taxa. Posterior probabilities from complete analysis are indicated at nodes on the Bayesian 50% majority rule tree.
Fig. 3  Stochastic character mapping of gland states associated with plant carnivory. Shown is stochastic character mapping of gland types and associated vasculature in SIMMAP using the 50% majority rule tree assembled from Bayesian inference phylogenetic reconstruction of the concatenated internal transcribed spacer, chloroplast intergenic spacer (PY-IGS), atpB, matK, petB, PTR1, rbcL, and trnK data set for 19 taxa. Pie charts at nodes represent ancestral states at each node that were calculated as the marginal posterior probability of each possible character state. Three basic gland types and associated vasculature found in carnivorous Caryophyllales as well as closely related noncarnivorous taxa were mapped: sessile glands (A), stalked glands (B), and pitted glands (C).
Ancistrocladaceae, and their presence is reconstructed as an ancestral character state (0.58 PP) for the clade containing Nepenthaceae and members of Ancistrocladaceae, Dioncophyllaceae, and Drosophyllaceae. However, in this case pitted glands are secondarily lost in Drosophyllaceae (currently absent in Drosophyllum) and the Dioncophyllaceae (0.90 PP). Pitted glands are nonvascularized in all taxa sampled in our analyses.

Discussion
Assessing Phylogenetic Relationships among the Carnivorous Caryophyllales

Bayesian and ML inference of phylogeny of the Caryophyllales and closely related taxa based on the combined molecular data set revealed a tree topology similar to that of previous molecular studies, which include a single-gene analysis of matK (Meimberg et al. 2000) and combined analyses of 18S, rbcL, atpB, and matK (Cameron et al. 2002).

The relationship presented here of a monophyletic Drosera with Drosera regia as the closest living ancestor to the remainder of sampled Drosera species is incongruent with previous matK and rbcL single-gene phylogenies yet is consistent with 18S, rbcL, atpB, and matK topologies presented in the same study (Cameron et al. 2002). Our analyses are also inconsistent with the findings of a rbcL study of primarily Drosera (Rivadavia et al. 2003), where Drosera was found to be polyphyletic as a result of D. regia and Aldrovanda forming a clade sister to the remaining Drosera species, with Dionaea sister to all remaining Droseraceae. A multiple-gene study (Cameron et al. 2002) is the most similar to our topology with regard to Droseraceae relationships; however, our eight-molecular-marker analysis provides higher support for the monophyly of Drosera (88 BS/1.00 PP), as well as the sister relationship between Dionaea and Aldrovanda (100 BS/1.00 PP). Our analyses are also inconsistent with the findings of an rbcL study of primarily Drosera (Rivadavia et al. 2003), in which Drosera was found to be polyphyletic as a result of D. regia and Aldrovanda forming a sister relationship just outside the entirety of sampled Drosera and Dionaea was found to be the closest living relative to all remaining Droseraceae.

Single-gene analyses of rbcL (Fay et al. 1997; Lledó et al. 1998) included sufficient taxa to test generic relationships across the carnivorous Caryophyllales. In Fay et al. (1997), Nepenthaceae was reconstructed as sister to a clade containing Droseraceae and members of the families Plumbaginaceae and Polygonaceae. The remaining families (Ancistrocladaceae, Dioncophyllaceae, and Drosophyllaceae) were recovered as sister to this clade. In Lledó et al. (1998), Nepenthaceae was recovered as sister to Droseraceae, and Drosophyllaceae was recovered as sister to a clade containing Drosophyllaceae, Dioncophyllaceae, and Ancistrocladaceae. The results of both analyses are inconsistent with the tree topology of our eight-molecular-marker analysis (fig. 1).

There has been uncertainty in the phylogenetic placement of Nepenthaceae in almost all previous analyses of the Caryophyllales (Fay et al. 1997; Nandi et al. 1998; Soltis et al. 2000; Cameron et al. 2002; Cuénoud et al. 2002). The exception is a single-gene analysis of matK (Meimberg et al. 2000) in which Nepenthaceae and a clade containing Ancistrocladaceae, Dioncophyllaceae, and Drosophyllaceae was sister to Droseraceae with moderate support. In our eight-molecular-marker analysis, Nepenthaceae as sister to the clade containing Ancistrocladaceae, Dioncophyllaceae, and Drosophyllaceae is highly supported (89 BS/1.00 PP). In addition, past analyses have shown little support for the reconstruction of Nepenthaceae and Droseraceae as sister clades (Fay et al. 1997; Lledó et al. 1998; Nandi et al. 1998; Soltis et al. 2000; Cuénoud et al. 2002; Hilu et al. 2003). In contrast, combined as well as ITS and PY-IGS analyses have shown strong support for Droseraceae as sister to the clade containing Nepenthaceae, Ancistrocladaceae, Dioncophyllaceae, and Drosophyllaceae (100 BS/1.00 PP).

Drosophyllum was previously thought to be allied with the Droseraceae (Cronquist 1988), an idea supported by an early rbcL analysis (Williams et al. 1994) that placed Drosophyllum sister to Drosophyllum. A later analysis of rbcL (Lledó et al. 1998) also suggested a relationship between Drosophyllum and Droseraceae; one of their three equally most parsimonious trees depicted Drosophyllum as sister to a clade that included Drosophyllum, Ancistrocladus, and Triphysophyllum. Since this time, Drosophyllum has been separated from Droseraceae and moved into the monotypic Drosophyllaceae on the basis of several multiple-locus phylogenies that suggest a sister relationship between Drosophyllum and the Nepenthaceae (APG II 2003). Our analyses clearly separate Drosophyllum from both Droseraceae and Nepenthaceae, placing it in a moderately well-supported clade with Ancistrocladus, Dioncophyllaceae, and Triphysophyllum (fig. 1). In our ITS and PY-IGS BI phylogenetic reconstruction (fig. 2), Drosophyllum forms a polytomy with Dioncophyllaceae and Triphysophyllum. The placement in the ITS and PY-IGS analysis is likely an artifact of the smaller data set not providing sufficient phylogenetically informative characters to resolve the relationship between Dioncophyllaceae, Drosophyllaceae, and Triphysophyllum.

The relationship of Ancistrocladus as sister to Dioncophyllaceae and Triphysophyllum has very high support (100 BS/1.00 PP), similar to the findings of previous analyses that recovered this relationship (Fay et al. 1997; Meimberg et al. 2000; Soltis et al. 2000; Cameron et al. 2002; Cuénoud et al. 2002; Hilu et al. 2003; Heubl et al. 2006). Meimberg et al. (2000) and Heubl et al. (2006) also recover Dioncophyllaceae as sister to Triphysophyllum, comparable to our results (100 BS/1.00 PP).

Topological incongruence observed among ours and previous phylogenetic reconstructions may be due to a sampling limitation of species per genus in previous studies and/or the amount of missing data in many of the combined molecular marker analyses (Wiens 2003). We tested for familial relationships through larger sampling of species within each genus, limiting the amount of missing molecular data, and using Bayesian and likelihood-based methods for phylogenetic reconstruction to decrease the potential for long-branch attraction (Bergsten 2005), especially considering the seemingly long evolutionary distances in some of the monotypic lineages (Drosophyllum, Dionaea, and Aldrovanda; figs. 1, 2). Because both our BI and ML analyses (ML branch lengths
are not shown in fig. 1) agree with the long branch associated with *Aldrovanda vesiculosa*, it is unlikely that BI's sometimes-inaccurate branch-length estimates are the cause (Brown et al. 2010). It is more likely that the long branch is an relic of extinction (Magallon 2010), given that *A. vesiculosa* represents a larger lineage with a rich fossil record dating back to the early Tertiary (Degraaf 1997).

**Ancestral Reconstruction of Carnivorous Glands of the Caryophyllales**

The results of our phylogenetic reconstructions provide a backbone to investigate the evolution of the carnivorous habit at the level of the gland—a morphological feature of the carnivorous plant trap that allows for the secretion of enzymes and absorption of digested products (Amagase 1972; Amagase et al. 1972; Dexheimer 1978; Henry and Steer 1985; Stoltzfus et al. 2002). The presence of glands is a synapomorphy for the noncore Caryophyllales (Judd et al. 2002). Glands can be either sessile, stalked, or pitted, and while sometimes vascularized with xylem and phloem, the presence of vasculature within a gland is not an indicator of its functionality in carnivory.

Stochastic mapping of gland morphology resulted in sessile gland type as being most likely ancestral when considering gland type alone, whether absent, sessile, stalked, or pitted (fig. 3A). Sessile glands containing xylem and phloem evolved independently in *Drosophyllum* and *Triphyophyllum*, a feature absent from all other sessile glands found in Droseraceae, Plumbaginaceae, and Polygonaceae (fig. 3A). Stalked and pitted glands are gained independently by ingroup and outgroup taxa, with lower posterior probability values for the occurrence of these glands as ancestral character states for *Limonium* and *Polygnum*. These results could be due to the lack of knowledge and inclusion of characters related to gland functionality in our analyses.

Results from stochastic character mapping imply that the evolution of stalked glands with xylem-containing vasculature (Droseraceae) and those that contain both xylem and phloem (*Drosophyllum* and *Triphyophyllum*) occurred as separate events (fig. 3B). In addition, according to these results it is also highly unlikely (0.12 PP) that the evolution of glands containing both xylem and phloem occurred in the common ancestor to the clade comprising *Ancistrocladaceae*, Dionophyllaceae, and *Drosophyllaceae*. Instead, the evolution of glands with both xylem and phloem was likely to have occurred twice, once in the lineage leading to the extant full-time carnivore *Drosophyllum* and a second time in the lineage leading to the part-time carnivore *Triphyophyllum* after the divergence of the *Dionocophyllaceae* lineage. Additional studies investigating the development and secretion chemistry of stalked glands in *Drosophyllum* and *Triphyophyllum* would help shed light on this apparent homoplasy.

Stochastic character mapping also demonstrates the loss of stalked glands by the common ancestor of the flytraps *Dionaea* and *Aldrovanda* (fig. 3B). These taxa are considered carnivorous and retain sessile glands without associated vasculature. It has been proposed that vascularized, stalked, multicellular glands may have been reduced to teeth and trigger hairs during the evolution of the lamina—a hypothesis with supporting evidence derived by Williams (1976) and revisited by Gibson and Waller (2009). This hypothesis is consistent with our reconstruction of ancestral gland character states, in which stalked glands have been lost in *Dionaea* while teeth and trigger hairs have been gained as a morphological character (data not coded). As in *Dionaea*, the teeth and trigger hairs of the *Aldrovanda* trap may be derived from vascularized stalked glands, resulting in the apparent loss of these glands. If so, this conversion of vascularized stalked glands to trigger hairs and teeth could have occurred in the common ancestor of *Dionaea* and *Aldrovanda*.

It is evident that the evolution of the vascularized gland is a novel feature of the carnivorous Caryophyllales, as only *Drosera*, *Drosophyllum*, and *Triphyophyllum* have vascularized glands involved in carnivory. In some genera of the Caryophyllales we found a lack of association between gland vasculature and carnivory (i.e., *Dionaea* and *Nepenthes*), as not all carnivorous plants have vascularized glands. It is also apparent that sessile and stalked glands are not required for carnivory in *Nepenthes*. Our investigations of gland morphology among carnivorous taxa and closely related non-carnivorous taxa therefore demonstrate that the internal architecture of glands on the lamina is not indicative of plant carnivory.

**On the Origin of Carnivorous Glands in the Caryophyllales**

It is clear that plant carnivory is independent of the presence of vascularized glands in *Dionaea*, *Aldrovanda*, and *Nepenthes* (fig. 3), as the absorption of nutrients by nonvascularized glands has been exemplified within *Dionaea*, *Aldrovanda*, and *Nepenthes* (Fabian-Galan and Salageanu 1968; Robins and Juniper 1980; An et al. 2002). This prompts two questions: how did vascularized glands arise in *Drosera*, *Drosophyllum*, and *Triphyophyllum*, and why did they arise?

Vascularized, stalked, multicellular glands that excrete enzymes and absorb nutrients for plant carnivory could have evolved by a number of methods, two of which could have been pinnation or emargination of the leaf blade (fig. 4). If pinnation, marginal glands were formed by homologous transformation of pinnae into vascularized stalked glands. Alternatively, emargination of the leaf blade could have occurred, whereby marginal glands evolved through gland definition. After the formation and evolution of vascularized, stalked, multicellular glands, the ancestors of extant carnivorous Caryophyllales diversified and radiated through modification or reduction of stalked glands (Juniper et al. 1989; Gibson and Waller 2009). During three separate events, stalked glands with vasculature were acquired by ancestors of Droseraceae, Drosophyllaceae, and Dionophyllaceae, leading to extant carnivorous taxa in the genera *Drosera*, *Drosophyllum*, and *Triphyophyllum*. Reduction of vascularized stalked glands is hypothesized to have occurred in ancestors of extant *Aldrovanda* and *Dionaea*, whereby vascularized stalked glands were reduced to vascularized teeth at the margin of the trap, as well as trigger hairs on the abaxial side of the leaf surface (Williams 1976; Gibson and Waller 2009). Stochastic character mapping for pitted, sessile, and stalked glands supports the loss of stalked glands at the ancestral node to *Dionaea* and *Aldrovanda* (fig. 3B).
Evidence to support pinnation, or the homologous transformation of pinnae to marginal glands, comes from studies of *Drosera* leaf development. In *Drosera capensis* seedlings, marginal glands that resemble pinnae can be found on cotyledons (Diels 1906). Interestingly, some closely related living members to the carnivorous Caryophyllales exhibit pinnately lobed leaves (e.g., Plumbaginaceae and *Limonium imbricatum*). If this type of leaf morphology is present in the Caryophyllales, it is possible that an ancestor to the carnivorous plants could have evolved pinnately compound leaves and secondarily evolved marginal glands through rearrangement of leaf architecture. Alternatively, emargination of the leaf blade occurred, and marginal glands evolved at the leaf margins. After the formation and evolution of stalked glands, the Caryophyllales diversified and radiated into the carnivorous taxa known today through retention or reduction of glands. Extant taxa have sessile, stalked, or pitted glands. Teeth and trigger hairs, two unique morphological features of *Dionaea* and *Aldrovanda*, respectively, may be homologous to stalked glands. The three separate vascularization events in ancestors of *Drosera*, *Drosophyllum*, and *Triphyophyllum* are indicated, and the presence of xylem and/or phloem is represented by x (xylem) and p (phloem).

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Appendix

Voucher Information

The voucher information includes the names of taxa used in this study, details on tissue collection, herbaria where the vouchers are deposited, and GenBank accession numbers for the sequences of ITS, PY-IGS, atpB, matK, petB, PTR1, rbcL, and matK. Greenhouse-grown specimens cultivated at the Botanical Garden of the University of California, Berkeley; California Carnivores; Missouri Botanical Garden; or elsewhere are noted after the voucher information: California Carnivores, Sebastopol, California (Peter D’Amato) = CC, Indonesian Institute of Sciences/Center for Plant Conservation–Bogor Botanical Gardens = LIPI/CPCBG, Missouri Botanical Garden = MO, Botanical Garden of the University of California, Berkeley = UCBG, and Botanischer Garten der Universität Würzburg = BGW. Voucher specimens are deposited in the following herbaria: Missouri Botanical Garden = MO, University and Jepson Herbaria of the University of California, Berkeley = UC, and Universität Würzburg = UW. Dashes indicate missing data.

**Taxon**; **ITS**, **PY-IGS**, **atpB**, **matK**, **petB**, **PTR1**, **rbcL**, **matK**; voucher specimen or living collection number; DNA collection number; collection locale; herbarium.

**Aldrovanda vesiculosa**; HM204865, HM204823, AY096108, AY096120, –, –, AY096106, –; TR174; USA, New Jersey, cultivated (R. Sivertsen); UC.

**Ancistrocladus abbreviatus**; HM204866, –, –, AF204840, FN598602, –, –, AF315939; 97–12-B-10; TR114; BGW, cultivated; UW. **Ancistrocladus barteri**; HM204867, HM204824, –, –, –, –, 05–87-B-10; TR118; BGW, cultivated; UW. **Ancistrocladus benomensis**; HM204868, HM204825, –, –, –, –, 00–54-B-20; TR117; BGW, cultivated; UW. **Ancistrocladus cochinchenensis**; HM204869, HM204826, –, –, –, –, 04–96-B-20; TR120; BGW, cultivated; UW. **Ancistrocladus congoensis**; HM204870, –, –, –, –, –, 00–29-B-10; TR119; BGW, cultivated; UW. **Ancistrocladus grandiflorus**; HM204871, HM204827, –, –, –, –, R.E. Gereau 5557; TR122; MO, cultivated; MO. **Ancistrocladus guineensis**; HM204872, HM204828, –, –, –, –, –, R.E. Gereau 5546; TR123; MO, cultivated; MO. **Ancistrocladus hamatus**; HM204873, HM204829, –, –, –, –, TR113; LIPI/CPCBG, cultivated; –. **Ancistrocladus beyneanus**; HM204874, HM204830, –, AF204841, –, –, –, –, GQ470529; 95–9–B-10; TR115, BGW, cultivated; UW. **Ancistrocladus korupensis**; BM204875, HM204831, AF209526, AF204839, –, –, Z97636, GQ470536; 99–3–B-10; TR116; BGW, cultivated; UW. **Ancistrocladus lesteii**; –, HM204832, –, –, –, –, –, R.E. Gereau 5566; TR123; MO, cultivated; MO. **Ancistrocladus robertsoniorum**; HM204876, HM204833, –, –, –, –, K.M. Meyer 277; TR124; MO, cultivated; MO.

**Dionaea muscipula**; HM204877, HM204834, AY096112, –, FN598597, –, DONCRPRBC, –; 2009.0254; TR186; UCBG, cultivated; UC.

**Dioncophyllum thalloni**; HM204878, HM204835, –, AF204844, –, –, –, –, –, G. Walters 1948; TR187; Gabon, Haut-Ogooué, Batéké Plateaux; MO.

**Drosera binata**; HM204879, HM204836, –, –, –, –, DRSCPRBC, –; 2001.0104; TR03; UCBG, cultivated; UC. **Drosera capensis**; HM204880, –, –, AY096122, –, –, –, DRSCPRBCL, –; 69.0172; TR05; UCBG, cultivated; UC. **Drosera dieliana**; HM204881, –, –, –, –, TR0004; TR41; CC, cultivated; UC. **Drosera falconeri**; HM204882, –, –, –, –, –, TR0073; TR112; CC, cultivated; UC. **Drosera graminifolia**; HM204883, –, –, –, –, TR0015; TR53; CC, cultivated; UC. **Drosera hamiltonii**; HM204884, –, –, –, –, TR0009; TR47; CC, cultivated; UC. **Drosera nidiiformis**; HM204885, –, –, –, –, –, TR0003; TR40; CC, cultivated; UC. **Drosera paleacea**; HM204886, –, –, –, –, –, TR0011; TR49; CC, cultivated; UC. **Drosera regia**; HM204887, HM204837, AY096111, AF204848, FN598596, –, DRSCPRBCG, –; TR0002; TR39; CC, cultivated; UC. **Drosera rotundifolia**; HM204888, HM204838, –, –, –, AB298084, –, AB072538, –; TR0024; TR62; CC, cultivated; UC. **Drosera slackii**; HM204889, –, –, –, –, –, TR0001; TR38; CC, cultivated; UC.

**Drosophyllum lusitanicum**; HM204890; HM204839, AY096113, AF204846, FN598600, –, DRHCPRBCLA, AY514860; TR0023; TR61; CC, cultivated; –.

**Limonium**; EU414036, –, AF209620, AY042610, FN598585, –, AF206789, AY514861; –, –, –.

**Nepenthes alata**; HM204891, HM204840, AF093388, AF204834, –, AF080545, NETCPBCL, AF315891; 87.0830; TR09; UCBG, cultivated; UC. **Nepenthes albomarginata**; HM204892, HM204841, –, –, –, –, 95.1376; TR10; UCBG, cultivated; UC. **Nepenthes boschiana**; HM204893, HM204842, –, –, –, –, 2004.0625; TR11; UCBG, cultivated; UC. **Nepenthes glandulifera**; HM204895, HM204844, –, –, –, –, 2005.1319; TR14; UCBG, cultivated; UC. **Nepenthes gracillima**; HM204896, HM204843, –, –, –, –, 95.1453; TR15; UCBG, cultivated; UC. **Nepenthes gymnophthora**; HM204897, HM204846, –, –, –, –, 95.1499; TR16; UCBG, cultivated; UC. **Nepenthes bursata**; –, HM204847, –, –, –, –, 93.0479; TR17; UCBG, cultivated; –. **Nepenthes insignis**; HM204898, HM204848, –, –, –, –, 95.1399; TR19; UCBG, cultivated; UC. **Nepenthes maxima**; HM204901, HM204851, –, –, –, 76.1342; TR22; UCBG, cultivated; UC. **Nepenthes mirabilis**; HM204902, HM204852, –, –, –, –, 95.1378; TR23; UCBG, cultivated; UC. **Nepenthes nortmannia**; HM204903,
HM204853, –, –, –, –, 95.1124; TR24; UCBG, cultivated; UC. Nepenthes rafflesiana; HM204904, HM204854, –, –, –, –, 95.1124; TR24; UCBG, cultivated; UC. Nepenthes reinwardtiana; HM204905, HM204855, –, –, –, –, 95.1297; TR27; UCBG, cultivated; UC. Nepenthes sinclairiana; HM204907, 95.1292; TR29; UCBG, cultivated; UC. Nepenthes spectabilis; HM204908, 95.1516; TR31; UCBG, cultivated; UC. Nepenthes tentaculata; HM204909, 95.1516; TR31; UCBG, cultivated; UC. Nepenthes sanguinea; TR26; UCBG, cultivated; –.

Nepenthes bilis

Nepenthes reinwardtiana –, –; 69.0037; TR25; UCBG, cultivated; UC.

Nepenthes rafflesiana; HM204903, –, –, –, –, –, –; 95.1124; TR27; UCBG, cultivated; UC.

Nepenthes sanguinea; TR26; UCBG, cultivated; –.

Nepenthes truncata

Nepenthes ventricosa


Drosera capensis

Drosera peltata

Triphyophyllum peltatum


Drosera capensis

Nepenthes rafflesiana

Nepenthes bilis

Drosera peltata

Triphyophyllum peltatum


Drosera capensis

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