Recent Progress in Understanding the Evolution of Carnivorous Lentibulariaceae (Lamiales)

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Abstract: Carnivorous plants have emerged as model systems for addressing many ecological and evolutionary questions, and since Lentibulariaceae comprise more than half of all known carnivorous species (325 spp.), they are of particular interest. Studies using various molecular markers have established that Lentibulariaceae and their three genera are monophyletic with *Pinguicula* being sister to a *Genlisea-Utricularia*-clade, while the closest relatives of the family remain uncertain. Character states of the carnivorous syndrome in related proto-carnivorous lamialean families apparently emerged independently. In *Utricularia*, the terrestrial habitat has been reconstructed as plesiomorphic, and an extension of subgenus *Polypompholyx* is warranted. In the protozoan-attracting *Genlisea*, subgenus *Tayloria* is revealed as basal lineage. In *Pinguicula*, the six major lineages found reflect radiations in clearly defined geographic regions, whereas most previously recognized subgeneric taxa are non-monophyletic. *Genlisea* and *Utricularia* exhibit substitutional rates that rank among the highest in angiosperms for the molecular markers analyzed. One possible explanation for this lies in selective constraints on a wide range of genomic regions that may have been lowered due to the use of an alternative mode of acquiring nutrients.

Key words: Carnivorous plants, Lentibulariaceae, *Utricularia*, *Genlisea*, *Pinguicula*, phylogeny, substitutional rates, molecular evolution.

Introduction

The Lentibulariaceae (bladderwort family) comprise approximately 325 species according to most recent diversity estimates (e.g., Barthlott et al., 2004; Fischer et al., 2004), with about 85 species currently distinguished in *Pinguicula*, 21 species of *Genlisea*, and an estimated 220 species in the largest carnivorous plant genus, *Utricularia*.

Of all genera in Lentibulariaceae, *Pinguicula* (the butterworts) arguably exhibits the most primitive trapping system, basically consisting of mucilage-covered leaves borne in a basal rosette, the margins of which can be rolled inwards (Fig. 1A). The most elaborate treatment of *Pinguicula* is the monograph of Casper (1966), while a number of later-described species were reviewed by Legendre (2000). A detailed phylogenetic treatment, however, was not available until very recently (Cieslack et al., 2005).

*Genlisea* (the corkscREW plants) is the smallest genus and has Y-shaped, twisted subterrestrial eel traps used to attract and trap soil protozoa (Barthlott et al., 1998) (Fig. 1B). Systematic treatments for the African (Fischer et al., 2000) and South American species (Fromm-Trinta, 1977, 1978, 1979, 1981; Fromm-Trinta and Taylor, 1985; Taylor and Fromm-Trinta, 1983) are available, but there has not been a more extensive phylogenetic treatment.

The largest genus, *Utricularia* (the bladderworts), exhibits the most complex trapping device, among the most complicated leaf modifications known in the plant kingdom. The bladder traps of *Utricularia* are either submerged (Fig. 1C) or subterrestrial and work by means of low pressure, and at least some of the terrestrial species have also been shown to trap protozoa or algae (Seine et al., 2002).

Here, we attempt to summarize recent studies that have addressed the evolution of Lentibulariaceae from a molecular phylogenetic perspective. We briefly discuss the state of knowledge on the closest relatives of the Lentibulariaceae among Lamiales, and argue that considerable work remains to be done before a convincing picture emerges. We further illustrate the current hypotheses on organismal phylogenies of each of the three genera, and point out inconsistencies between gene trees or where a more extensive sampling will be required to obtain further insights. Finally, we extend our considerations to the molecular evolutionary level by discussing alternative hypotheses put forward to explain remarkably increased DNA substitution rates in *Utricularia* and *Genlisea*.

Phylogenetic Relationships in Lamiales and the Closest Relatives of Lentibulariaceae

All phylogenetic studies hitherto conducted in Lamiales that sampled more than one representative of Lentibulariaceae have identified the family as monophyletic (Albach et al., 2001; Albert et al., 1992; Bremer et al., 2002; Jobson et al., 2003; Müller et al., 2004; Olmstead et al., 2001; Olmstead...
Various hypotheses have been put forward on the closest relatives of Lentibulariaceae in Lamiales, but none have received significant statistical support. The close proximity of proto-carnivorous genera in Lamiales (Byblis, Ibicella) was considered the most parsimonious scenario, requiring only one single emergence of adaptations that are key to later expression of the full carnivorous syndrome (pre-adaptations). Byblis was reconstructed as closest relative in an early analysis of rbcl (Albert et al., 1992), though statistical support was lacking. Other studies using more comprehensive sampling of the order Lamiales arrived at different candidates from within Lamiales, e.g., Hédren et al. (1995) and Oxelman et al. (1999). Such studies highlighted the effects of taxon sampling and hypotheses based upon differing inference methods (Bignoniaceae vs. Lamiaceae, Müller et al., 2004; Rahmanzadeh et al., 2005) (Fig. 2). Byblis was seen as closest relative in most studies (Albert et al., 1992; Bremer et al., 2002; Jobson et al., 2003), a fact that may have led to its inclusion in the Lentibulariaceae in recent APG classifications (APG2, 2003). However, there is general agreement that the phylogenetic “backbone” of the order Lamiales is still far from being understood, and the interrelationships of most of the formerly accepted families in the order continue to be unclear (Albach et al., 2005; Ghebrehiwet et al., 2000; Hoggard et al., 2003; Olmstead et al., 2001; Oyama and Baum, 2004; Wolfe et al., 2002).

A recent analysis of matK could not establish the closest relatives with confidence and, using topological tests, rejected the scenario of proto-carnivorous genera in Lamiales (Byblis or Ibicella) being closest relatives. In consequence, the scenario of a single evolutionary gain of key components of the carnivorous syndrome in the order was rejected, and a parallel acquisition of pre-adaptations to carnivory was favoured by the data (Müller et al., 2004). Differences in gland morphology support parallel acquisition of carnivorous traits in Byblis (Byblidaceae), Ibicella (Martyriaceae), and Lentibulariaceae. Digestive glands in all three genera of Lentibulariaceae are attached to vessels, unlike secretory glands of Byblidaceae and Martyriaceae that rest on at least two epidermal cells.
Accordingly, much work remains to be done before a satisfactory identification of the closest relatives of Lentibulariaceae in Lamiales is possible. A series of studies recently identified new lineages in the order and invalidated original/old taxonomic concepts. First, the traditional circumscription of the family Scrophulariaceae had to be abandoned (Olmstead et al., 2001; Olmstead and Reeves, 1995; Oxelman et al., 2005; Reeves and Olmstead, 1998). Later, a new circumscription for Plantaginaeae emerged (Albach et al., 2005; Bello et al., 2002; Hoggard et al., 2003; Roested et al., 2002) and morphology and DNA data allowed the delimitation of new families such as Linderniaceae (Rahmanzadeh et al., 2005), whereas former tribes of Scrophulariaceae continue to be elucidated (Albach et al., 2004; Ghebrehiwet et al., 2000; Wolfe et al., 2002). While Lamiales have long been known to be notoriously difficult to resolve with common DNA markers (Albach et al., 2001; Olmstead et al., 1992, 2000, 2001; Olmstead and Reeves, 1995), non-coding markers have proved to have particularly promising properties (Borsch et al., 2005; Müller et al., 2006; Olmstead et al., 1998) for resolving lineages in the order, and first analyses of rapidly evolving chloroplast DNA markers have already improved resolution in parts of the Lamiales tree (Bremer et al., 2002; Rahmanzadeh et al., 2005). Further work in that direction is underway.

Relationships Among Genera and Evolution of Carnivory in the Family

All studies conducted so far concur in that the three genera are clearly monophyletic and that Pinguicula branches first as sister to a clade consisting of Utricularia and Genlisea (Cieslack et al., 2005; Jobson et al., 2003; Müller and Borsch, 2005; Müller et al., 2000, 2004).

After screening the morphology of the various gland types in Lentibulariaceae and other families of Lamiales, it appears that one of the key inventions of the most recent common ancestor (MRCA) of Lentibulariaceae was absorptive glands attached to tracheid elements, consisting of one epidermal cell supporting an endodermal cell and glandular head cells occurring in multiples of two (Müller et al., 2004). By means of digestive enzymes, the glands can absorb organic substances such as amino acids and nucleotides that are released during the decomposition of insects and other organic matter (Dixon et al., 1980; Lüttge, 1983), thus supplementing the energetically expensive reduction of nitrogen oxides by direct uptake of N-containing organic molecules (e.g., amino acids) and ammonium from prey. Such compounds were previously unavailable caused by these glands.

This MRCA was further reconstructed and shown to have possessed a basal rosette composed of flat leaves and a primary root, reduced soon after germination (Müller et al., 2004). A rosette was later lost with the adaptation to aquatic habitats in a terminal lineage of Utricularia. While in the Pinguicula lineage adventitious roots were maintained, root functions were taken over by the leaves or shoots in Utricularia and Genlisea, and roots were lost in both genera (Brugger and Rutishauser, 1989; Lloyd, 1942; Taylor, 1989).

The MRCA of Utricularia and Genlisea developed inverse tropism and episcidiate leaves, with abaxially closed leaf margins. Indeed, traps in extant Utricularia and Genlisea are morphologically regarded as highly modified episcidiate leaves (Lloyd, 1942; Rutishauser and Sattler, 1989). Also, mutants with episcidiate pitchers instead of flat leaves are known from various angiosperms, including Plantago and Pinguicula (Barthlott et al., 2004; Juniper, 1986), suggesting a narrow genetic basis for this mutation. Similarly, inverse tropism is not uncommon in plants, as exemplified by pendent epiphytic life forms. In Lentibulariaceae, it has been suggested that the selection pressure to reverse tropism may be sought in the rich and probably more regular nutrient supply encountered in the soil (Müller et al., 2004), with an abundance of protists and other microscopic organisms (Barthlott et al., 1998; Seine et al., 2002).

Highly enhanced substitutional rates in all genomes found in Utricularia and Genlisea are likely to have played a major role in the evolution of this lineage (Jobson and Albert, 2002; Müller et al., 2004; see below).

Biogeography Correlates Well with Major Clades in Pinguicula

Pinguicula is the second most diverse genus of the Lentibulariaceae, with 85 currently accepted species (Legendre, 2000). Here, insects are caught and digested by sessile and short-stalked mucilage-producing glands on the upper side of rosette leaves forming so-called “flypaper traps”. The common name “butterworts” is derived from the shiny leaf surface caused by these glands.

Phylogenetic relationships in Pinguicula were recently elucidated by Cieslack et al. (2005). Fig. 3 strongly simplifies the results of this study based on 43 species of Pinguicula. Most previously recognized subgenera and sections of the genus (Casper, 1966; reviewed and supplemented by Legendre, 2000) were shown to be para- or polyphyletic. A notable exception is section Pinguicula, which is monophyletic. However, the identified lineages largely reflect radiations in clearly defined geographic regions.

All Central American, Mexican, and Caribbean species (clade 1 in Fig. 3) were resolved in a clade sister to the Eurasian Pinguicula alpina. This surprising relationship was also supported by indels, but no morphological synapomorphy is currently
known for these two clades. Clade 1 disagrees with most previous infrageneric classifications, with the exception of De Candolle’s (1844) concept of section *Orcheosanthus*.

An East Asian clade was identified as successive sister to this clade of Central American, Mexican, and Caribbean species. A morphological synapomorphy for these East Asian species is peduncles that are densely covered by mucilage glands. All three clades together are characterized by the production of flower buds on winter-resting plants, a specific corolla hair structure, and a very large corolla lower central lobe.

Another diverse clade is composed of species with primarily European distribution, including the widespread type species *Pinguicula vulgaris*. For this clade, vegetative reproduction during dormancy is synapomorphmic.

Species native to SE North America and the South American Andes and a group of Mediterranean and NE Atlantic coast species together appear in another well-supported clade that is characterized by a particular growth type. It is the only clade that has reached temperate zones of the southern hemisphere.

Among the more obvious taxonomic changes that are suggested by the results of the study of Cieslack et al. is that *Pinguicula moranensis*, currently in section *Orcheosanthus* (Casper, 1966), should be included in section *Longitubus* together with *Pinguicula laueana*, with which it shares a conspicuously long spur.

The evolution of morphological traits will be discussed in more detail in another paper of this issue.
the differences in capsule dehiscence found between their members. While in subgenus Tayloria capsules open septically, subgenus Genlisea displays a unique circumscissile dehiscence type, with capsules sometimes opening spirally (illustrated in Stopp, 1958). In addition, Fischer et al. (2000) suggest three groups for the African species. These groups were distinguished based on floral characters and indumentum characteristics and were not assigned a taxonomic rank.

The first phylogenetic studies including at least a few species of Genlisea were those of Müller et al. (2004, six spp.) and Jobson et al. (2003, five spp.). These studies infer two clades reflecting subgenera Tayloria and Genlisea, respectively. The tree in Müller et al. (2004) also incorporated a representative of each of the three distinguished African groups, and points towards the non-monophyly of these African species, with the Madagascan and East African G. margaretae showing affinities to the neotropical species.

Extending the taxon sampling of Müller et al. (2004) with additional sequences of G. pygmaea, G. repens, and G. guianensis (Worberg et al., unpublished data; data matrix available upon request) provides the preliminary picture provided in Fig. 4. More sequences from additional taxa are currently being compiled and a detailed study, including a morphological survey of trap morphology using scanning electron microscopy, is underway and will be published elsewhere.

Phylogeny of Utricularia

Among carnivorous plants, the bladderwort Utricularia stands out for a variety of reasons. There are conspicuous modifications in vegetative morphology, with a body plan that exhibits out for a variety of reasons. There are conspicuous modifications in vegetative morphology, with a body plan that exhibits...

To summarize the phylogenetic hypotheses as a supertree, a matrix representation with parsimony (MRP, Baum, 1992; Ragan, 1992) was used here. Binary matrices reflecting the nodes present in trees from either study, with characters weighted by the statistical support of each node, were constructed with help of r8s (Sanderson, 2003), following recommendations in Bininda-Emonds and Sanderson (2001) and Salamin et al. (2002). Tree topologies and jackknife proportions were exported from TGF files to r8s-readable Newick format using TreeGraph (Müller and Müller, 2004). The MRP analysis resulted in...
The vast majority of the 34 sections distinguished by Taylor (1989) are monophyletic, with the following notable exceptions. Müller and Borsch (2005) established the paraphyly of section Iperua P. Taylor, since Utricularia humboldtii Schomb. is unequivocally supported as member of a clade comprising species of section Orchidioidea ADC. The resulting enhanced section Orchidioidea is remarkable for its representatives with relatively large, orchid-like flowers (hence the name).

All current evidence suggests that subgenus Utricularia is paraphyletic. This is because section Pleiochasia is resolved as closest relative of subgenus Polypropophylyx. The classification by Taylor into two subgenera Utricularia and Polypropophylyx was therefore suggested to be modified to better reflect phylogenetic relationships (Müller and Borsch, 2005). Subgenus Polypropophylyx was redefined to also include section Pleiochasia. Subgenus Utricularia was proposed to be narrowed down to include most of the aquatic and epiphytic sections (Fig. 5). The old subgeneric name Bivalvaria has been proposed to be restored to circumscribe all remaining taxa (Fig. 5). The respective clade, however, receives less than the average node support in the Utricularia tree (72% with trnK, 70% with trnLF/rps16). The relationship between the three subgenera remains largely unresolved. While Polypropophylyx is sister to Bivalvaria based on trnK (88% support), it is sister to the remainder of Utricularia using trnLF/rps16 (93%). Taking 95% confidence intervals into account, given the number of replicates used in both studies, the support for the conflicting node found with trnLF/rps16 is significantly higher (though close to insignificance at alpha = 0.05). Enhanced taxon sampling and use and combination of more data will help to resolve the remaining conflict with respect to the Utricularia “backbone” topology.

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According to both studies, section Setiscapella is paraphyletic to Vesculinia, and Psyllosperma is paraphyletic to Foliosa, which led Müller and Borsch (2005) to a new circumscription of the section with the older names, namely Vesculinia and Foliosa.

Jobson et al. (2003) revealed the non-monophyly of sections Oligocista (with U. nana of section Benjaminiinaa nested within Oligocista) and Utricularia (with U. olivacea showing affinities to section Vesculinia). These relationships could not be confirmed by trnK, most likely due to differences in taxon sampling. With inclusion of other representatives of Vesculinia, the latter section shows affinities to Setiscapella rather than Utricularia. Setiscapella itself appears clearly paraphyletic to Avesicaria in the trnL-F/rps16 tree, so Avesicaria is used in Fig. 5.

The situation remains unresolved so far with respect to section Aranella. Both studies incorporated a mutually exclusive set of representatives of this section, and both found that the species clustered together, but in different parts of the Bivalvaria clade. In the trnLF/rps16 tree, Aranella appears as sister to the remainder of Bivalvaria, though only supported with 58% JK. In the analysis of trnK/matK, it branches next in a series after Lloydia and Calpidisca. Complementary inclusion of the missing Aranella species in the trnL-F/rps16 and matK datasets is likely to reveal that this section is also polyphyletic.

All current evidence suggests that the ancestral life form of Utricularia was terrestrial. Epiphytes and lithophytes are derived within the genus, and independently occurred in sections Orchidioidea and Phyllaria (Jobson et al., 2003; Müller and Borsch, 2005).
Also, aquatics occupy derived positions within the Utricularia phylogeny. It appears that a parallel evolution of suspended plants (in sect. Vesiculina and independently Utricularia) took place, and that affixed aquatics can be considered intermediates in a transgression from terrestrials to free-floating, suspended aquatics (Jobson et al., 2003; Müller and Borsch, 2005). Interestingly, there also appears to be a parallel evolution of rheophytes (plants living in waterfalls or swiftly flowing water) from terrestrials, once in section Avesicarioides (paletropical; Müller and Borsch, 2005), and independently in the neotropical section Avesicaria (Jobson et al., 2003).

Current estimates concur in identifying South America as the geographic origin of Utricularia, taking the highly likely neotropical origin of the sister genus Genlisea into account (Jobson et al., 2003; Müller and Borsch, 2005).

High Evolutionary Rates in Utricularia and Genlisea

DNA substitution rates were found to be significantly higher in Genlisea and Utricularia compared to Pinguicula, based on a variety of relative rate test variants (using step distributions, distance-based and likelihood ratio tests) and several loci representing all three genomic compartments (Jobson and Albert, 2002; Müller et al., 2004). Moreover, comparing rates in chloroplast trnL sequences across 375 angiosperm genera, rates in the rootless, protozoa-trapping Utricularia-Genlisea lineage rank among the highest in angiosperms (Müller et al., 2004; Müller et al., submitted), slightly exceeded only by the similarly rootless, morphologically highly aberrant aquatic Podostemaceae. A parallel data matrix of rbcl sequences shows the same trend, though less statistical significance in view of lower overall rates in this genomic region (Müller et al., submitted).

A positive correlation of species diversity and branch lengths has been reported for angiosperms (Barraclough and Savolainen, 1996, 2001; Savolainen and Goudet, 1998) and was also used to explain longer branches found in the species-rich genus Utricularia versus Pinguicula, which contains less species (Jobson and Albert, 2002). In these studies, cladogenesis or diversification contrasts were assumed whenever one lineage comprised more species than the other. However, stochastic differences in initial rates can lead to a much higher diversity in one of the lineages (Slowinski and Guyer, 1989). We applied the standard null model test (Slowinski and Guyer, 1993) to assess the significance of species diversity contrasts. This test highly significantly rejects ($p = 0.528$; $p > 0.05$) amplified diversification in Utricularia (220 spp.) and Genlisea (21 spp.) as compared to the sister group Pinguicula (86 spp., Barthlott et al., 2004; Cieslack et al., 2005). Additional analyses, using for example tree imbalance (Kirkpatrick and Slatkin, 1993) or more sensitive three-taxon tests (Sanderson and Donoghue, 1994, 1996), are unlikely to change the basic conclusion of insignificant differences in diversification rates. Note that even in the most species-rich angiosperm genus, Astragalus, comprising five to ten times the number of species in all hypothesized sister groups, no higher diversification rates were found using three different analytic approaches ($p$ always $> 0.3$, Sanderson and Wojciechowski, 1996).

On the other hand, the currently available data indicate a trend towards high rates in lineages where the normal uptake of inorganic nutrients via roots is accompanied or replaced by some other mode of acquiring nutrients. It can be hypothesized that organic molecules, such as amino acids, play a substantial role in the nutrition of Genlisea and Utricularia and can be used directly in various metabolic pathways, resulting in a decreased need for nitrate reduction or transamination (Müller et al., 2004). As a consequence, selective constraints on a wide range of genomic regions could have been lowered. This does not preclude overlapping effects of differences in polymerase fidelity or repair systems, which so far have not been investigated (Müller et al., submitted).

Instead of relaxed constraints, positive selection may underlie lineage-specific changes in substitutional rates, such as in the highly interesting case of coxl, where rate shifts have been related to adaptive evolution of cytochrome c oxidase that may have been key to the successful radiation of Utricularia (Jobson et al., 2004).

According to these insights, a highly unconventional molecular evolutionary mode is evident in bladderworts and cork-screw plants. Interestingly, this is further underlined by the finding of extremely low genome sizes in these plants (see Greilhuber et al., 2006).

Conclusions

As yet, our understanding of the evolution of Lentibulariaceae is still incomplete. Although relationships between the genera are clearly unambiguous, and those within Pinguicula and Utricularia appear to be largely resolved, the closest relatives of the family still require substantiation. The same is true for the relationships within Genlisea and those of certain crucial, “difficult-to-sample” representatives of the other two genera. However, the data collected so far and the hypotheses put forward testify to the strong potential of extremely specialized lineages of organisms to provide insights into more fundamental mechanisms involved in molecular and character evolution. Interestingly, and in support of this statement, fundamental biological mechanisms have previously first been unravelled by investigation of carnivorous plants, such as the function of the Golgi apparatus (Schneff, 1960, 1961). Further studies on rather extreme model systems like Lentibulariaceae, with amazing alterations in biochemical or morphological characters, will be an invaluable complement to the in-depth analysis of the few model organisms.

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