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Evolution of Carnivory in Lentibulariaceae and the Lamiales

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Abstract: **As a basis for analysing the evolution of the carnivorous syndrome in Lentibulariaceae (Lamiales), phylogenetic reconstructions were conducted based on coding and non-coding chloroplast DNA (*matK* gene and flanking *trnK* intron sequences, totalling about 2.4 kb). A dense taxon sampling including all other major lineages of Lamiales was needed since the closest relatives of Lentibulariaceae and the position of "proto-carnivores" were unknown. Tree inference using maximum parsimony, maximum likelihood and Bayesian approaches resulted in fully congruent topologies within Lentibulariaceae, whereas relationships among the different lineages of Lamiales were only congruent between likelihood and Bayesian optimizations. Lentibulariaceae and their three genera *(Pinguicula, Genlisea,* and *Utricularia)* are monophyletic, with *Pinguicula* being sister to a *Genlisea-Utricularia* clade. Likelihood and Bayesian trees converge on Bignoniaceae as sister to Lentibulariaceae, albeit lacking good support. The "proto-carnivores" (Byblidaceae, Martyniaceae) are found in different positions among other Lamiales but not as sister to the carnivorous Lentibulariaceae, which is also supported by Khishino-Hasegawa tests. This implies that carnivory and its preliminary stages ("proto-carnivores") independently evolved more than once among Lamiales. Ancestral states of structural characters connected to the carnivorous syndrome are reconstructed using the molecular tree, and a hypothesis on the evolutionary pathway of the carnivorous syndrome in Lentibulariaceae is presented. Extreme DNA mutational rates found in *Utricularia* and *Genlisea* are shown to correspond to their unusual nutritional specialization, thereby hinting at a marked degree of carnivory in these two genera.**

Key words: **Carnivorous plants, Lamiales, Lentibulariaceae, *matK-trnK,* molecular phylogeny, mutation rates.**

Introduction

Carnivorous plants have fascinated scientists ever since Darwin's treatise in 1875 (Darwin, 1875), but surprising facts about these extraordinary plants are still being revealed. Recently, protozoa trapping plants were discovered (Barthlott et al., 1998) and others were found to feed on algae, thus being plant eaters (Seine et al., 2002). These specialists, *Utricularia* and *Genlisea,* possess tiny traps that rank among the most complex organs in the plant kingdom (Juniper et al., 1989) and belong to the family Lentibulariaceae (order Lamiales), the most species-rich carnivorous plant family. Beyond trapping prey, carnivory involves digestion and absorption of released nutrients. The selective advantage over non-carnivorous plants has been generally appreciated to be in an additional supply of macronutrients (N, P, S) (Juniper et al., 1989). In angiosperms, carnivorous plants have evolved in several different lineages (Albert et al., 1992): in the Poales *(Brocchinia, Catopsis),* Oxalidales *(Cephalotus),* Caryophyllales (e.g., venus fly trap *[Dionaea],* sundew *[Drosera],* pitcher plant *[Nepenthes]*), Ericales (e.g., *Sarracenia, Darlingtonia*), and Lamiales (the butterwort genus *Pinguicula,* the bladderwort genus *Utricularia,* and *Genlisea*). Within the Lamiales, the Australian Byblidaceae and the American Martyniaceae display some of the features of the carnivorous syndrome, but lack digestive enzymes and, therefore, have been referred to as "proto-carnivores" (Juniper et al., 1989). As a consequence, one might assume that these "proto-carnivores" are close relatives of Lentibulariaceae, with the carnivorous syndrome less developed. The cosmopolitan Lentibulariaceae (310 spp.) are all true carnivores and comprise three genera, *Pinguicula* (Casper, 1966), *Genlisea* (Fromm-Trinta, 1977), and *Utricularia* (Taylor, 1989). Whereas butterworts (*Pinguicula,* 74 spp.) trap insects with sticky flypaper traps, the species of *Genlisea* (21 spp.) attract protozoa in the soil, using Y-shaped, twisted organs (eel traps; Barthlott et al., 1998). *Utricularia* is the most diverse genus (215 spp.) and includes terrestrials, epiphytes, and aquatics. Its submerged or subterrestrial bladder traps work by means of low pressure, and at least some of the terrestrial species were also shown to trap protozoa (Seine et al., 2002).

In order to understand the origin and evolution of the carnivorous syndrome in Lamiales, a sound knowledge of the phylogenetic relationships of and among Lentibulariaceae is needed. Based on a phylogenetic tree, evolution of characters and their states can then be reconstructed. Compared to other angiosperm orders, the illumination of phylogenetic relationships in the Lamiales has proven particularly difficult in previous studies, regardless of whether single or combined genes were used (e.g., Olmstead et al., 2001; Olmstead and Reeves, 1995; Oxelman et al., 1999; Soltis et al., 2000). In these studies, however, only parsimony reconstruction has so far been applied,

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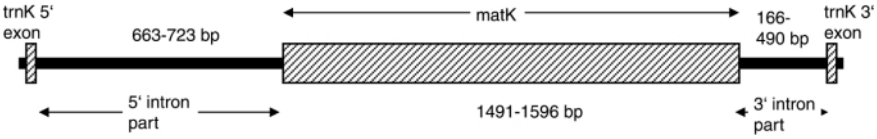

Fig. 1 The sequenced *trnK* intron, showing the relative size of the two noncoding intron parts (black bar), the *matK* coding region, and the *trnK* exons (hatched boxes). Numbers correspond to the size range (in bp) of each region found in the Lamiales data set.

which is much more sensitive to branch length heterogeneity than likelihood-based methods (Lewis, 1998; Swofford et al., 1996). Moreover, mostly rather slowly evolving genes, such as *rbcL* and *18S,* were sequenced. Only a recent combined analysis of numerous markers, including non-coding DNA (Bremer et al., 2002), has yielded a noteworthy improvement in resolution in the Lamiales, while still not allowing inference of the closest relatives of Lentibulariaceae with any degree of confidence.

The aims of this study are to infer the major clades within the Lentibulariaceae and to test the phylogenetic position of Lentibulariaceae within Lamiales, in particular the relationships of Lentibulariaceae to the "proto-carnivores". Here, we use the chloroplast *trnK* intron (Fig. **1**) for phylogenetic analysis, thus combining information from two non-coding (the 5′ and 3′ intron parts) and one coding region (the *matK* gene) for a representative sampling of Lentibulariaceae (63 taxa) and other Lamiales (24 taxa). The chloroplast *matK* gene is located within the *trnK* intron and is believed to code for a maturase, involved in the self-splicing of group II introns (e.g., Neuhaus and Link, 1997■; Mohr et al., 1993; Vogel et al., 1997). It is a fast evolving gene that has been shown to contain phylogenetic signal at different taxonomic levels (e.g., Hilu and Liang, 1997; Hilu, Müller and Borsch, unpubl. data; Hilu et al., 2003■; Johnson and Soltis, 1995). Different approaches for phylogeny reconstruction are employed (Maximum Parsimony, Maximum Likelihood, Bayesian Inference) to assess their effect on the resulting trees. This is particularly important since each of these tree reconstruction approaches relies on different preassumptions that, as such, cannot be falsified. Bayesian analysis is the most recent of the three methods, and compared to Maximum Likelihood, has the advantage that node evaluation becomes computationally feasible. Moreover, a set of morphological and anatomical characters related to the carnivorous syndrome are optimized on the *trnK/matK* tree that was found following a parsimony criterion, in order to obtain insights into the evolution of this syndrome. Since accelerated DNA mutational rates have been observed particularly in *Genlisea* and *Utricularia,* these rates are analyzed with respect to the evolution of carnivory in Lentibulariaceae.

Materials and Methods

Plant samples

Our data set comprised 31 species of *Utricularia,* 6 of *Genlisea,* and 26 of *Pinguicula.* The majority of sections currently distinguished in *Utricularia* (Taylor, 1989), all groups of morphologically allied species of *Genlisea* (Fischer et al., 2000), and all sections of *Pinguicula* recognized by Casper (1966) are represented. Solanales served as outgroup. The included species, vouchers and GenBank accession numbers are shown in Table **1**. Voucher specimens are deposited at the herbarium Bonn, unless otherwise specified.

DNA isolation, amplification, and sequencing

DNA was isolated from fresh or silica gel dried tissues using a modified CTAB buffer method (Borsch et al., 2003). To avoid contamination by DNA from trapped insects, flowers and peduncles were used, in particular for species of *Utricularia* and *Genlisea.* Purification of genomic DNA was achieved using QiaQuick columns (Qiagen Inc., Valencia, California). PCR reactions were performed in 25 µl-reactions containing 1 U *Taq* DNA polymerase (Promega), 1 mM dNTP mix (1.25 mM each), 1 × buffer (Promega), 1.5 mM $MgCl_2$, and 0.4 µM of each amplification primer. The PCR profile was 1:30 min at 96 °C, 1 min at 50 °C, 1:30 min at 72 °C, 35 cycles of 0:30 min at 95 °C, 1 min at 50 °C, 1:30 min at 72 °C, and a final extension of 10 min at 72 °C. In most taxa, the region was amplified in two overlapping halves. Primers used for amplification and sequencing are shown in Table **2**. Due to the high sequence variability in *Utricularia* and *Genlisea,* a high number of specific primers had to be designed. PCR products were electrophoresed in a 1.2% agarose gel and the appropriate bands were excised and subsequently purified using the QiaQuick gel extraction kit (Qiagen, Inc., Valencia, California). For cycle sequencing, the ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit with Amplitaq DNA polymerase FS (PE Applied Biosystems) was used according to the manufacturer's protocol. Electrophoresis was carried out on ABI 310 and ABI 377 automated DNA sequencers (PE Applied Biosystems).

Alignment and phylogenetic analyses

Phylogenetic analyses were conducted using *trnK* intron sequences of 89 species representing all major groups of species in the Lentibulariaceae and families in the Lamiales (Table **1**), and two members of family Solanaceae as outgroup. Sequences were aligned in QuickAlign (Müller and Müller, 2003). Alignment was easily achieved by eye, after initial use of ClustalX (Thompson et al., 1997). In noncoding parts of the *trnK* intron, length mutations were easily identified, mostly as simple sequence repeats following rules described in Kelchner (2000) and Borsch et al. (2003). In the analyses, gaps were treated as missing characters, and all characters were given equal weight. Maximum parsimony analyses (MP) were conducted using PAUP* (Swofford, 1998) and PRAT (Müller, 2002). PRAT generates command files that allow parsimony ratchet searches (Nixon, 1999) with PAUP*. The ratchet approach was used to ensure that the shortest trees are found since the dataset is comparably large. The searches were carried out using 10 random addition cycles of 200 ratchet iterations. The iterations comprised two rounds of TBR, one on a randomly reweighted data set, and the other on the original matrix, saving one shortest tree. 25% of the positions were randomly upweighted. A strict consensus tree was computed from the shortest trees collected from the different tree islands. Parsimony jackknifing (Farris et al., 1996) with 1000 replicates was carried out with TBR branch swapping on 10 saved

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Table 1 Taxa used in the present study, name of clade to which the taxa (usually species) are assigned, voucher information (vouchers are deposited in the herbarium BONN unless a different herbarium is noted in brackets), GenBank accession numbers, and references. The classification follows Taylor (1989) for *Utricularia*, Fromm-Trinta (1977) for *Genlisea*, Casper (1966) and Legendre (2000) for *Pinguicula*, and Bremer et al. (2001■) for families in the Lamiales

Species	Section/family	Name of clade in Fig. **3**	Voucher	GenBank accession number	Authors
Lentibulariaceae					
Pinguicula agnata Casper	*Agnata*	*P. sharpii + allies*	L. Legendre 1021 (NSW)	AF531782	this study
Pinguicula filifolia Wright ex Griseb.	*Agnata*	*P. filifolia + allies*	L. Legendre 1022 (NSW)	AF531786	this study
Pinguicula gigantea Luhrs	*Agnata*	*P. sharpii + allies*	L. Legendre 1023 (NSW)	AF531789	this study
Pinguicula rotundiflora Studnicka	*Heterophyllum*	*P. filifolia + allies*	L. Legendre 1028 (NSW)	AF531802	this study
Pinguicula sharpii Casper and Kondo	*Isoloba*	*P. sharpii + allies*	L. Legendre 1024 (NSW)	AF531803	this study
Pinguicula alpina L.	*Micranthus*	*P. alpina*	J. Steiger s.n. (JE)	AF531783	this study
Pinguicula moctezumae Zamudio Ruiz and Ortega	*Orcheosanthus*	*P. moctezumae*	L. Legendre 1025 (NSW)	AF531797	this study
Pinguicula moranensis H. B. K. var. *huahuapan*	*Orcheosanthus*	*P. moranensis + allies*	L. Legendre s.n. (NSW)	AF531799	this study
Pinguicula moranensis H. B. K. var. *moranensis*	*Orcheosanthus*	*P. moranensis + allies*	L. Legendre 1029 (NSW)	AF531798	this study
Pinguicula rectifolia Speta and Fuchs	*Orcheosanthus*	*P. moranensis + allies*	L. Legendre 1031 (NSW)	AF531801	this study
Pinguicula corsica Bern. and Gren. ex Gren. and Godr.	*Pinguicula*	*P. vallisneriifolia + allies*	J. Steiger 18 (JE)	AF531784	this study
Pinguicula fiorii Tammaro and Pace	*Pinguicula*	*P. vulgaris + allies*	J. Steiger 45 (JE)	AF531787	this study
Pinguicula fontiqueriana Romo, Peris and Stübing	*Pinguicula*	*P. vulgaris + allies*	J. Steiger s.n. (JE)	AF531788	this study
Pinguicula grandiflora Lam.	*Pinguicula*	*P. vulgaris + allies*	J. Steiger 7b (JE)	AF531791	this study
Pinguicula leptoceras Rchb.	*Pinguicula*	*P. leptoceras + allies*	J. Steiger 30a (JE)	AF531792	this study
Pinguicula longifolia Ram. ex Dc. ssp. *caussensis* Casper	*Pinguicula*	*P. vulgaris + allies*	J. Steiger 20 (JE)	AF531793	this study
Pinguicula longifolia Ram. ex Dc. ssp. *longifolia*	*Pinguicula*	*P. vulgaris + allies*	L. Legendre 1032 (NSW)	AF531794	this study
Pinguicula macroceras Link in Sprengel, Schrader and Link	*Pinguicula*	*P. vulgaris + allies*	J. Steiger 29 b (JE)	AF531796	this study
Pinguicula macroceras Link in Sprengel, Schrader and Link ssp. *nortensis* Steiger and Rondeau	*Pinguicula*	*P. vulgaris + allies*	L. Legendre 1033 (NSW)	AF531795	this study
Pinguicula mundi Blanca, Jamilena, Ruiz-Rejòn and Zamora	*Pinguicula*	*P. vulgaris + allies*	J. Steiger 22 (JE)	AF531800	this study
Pinguicula poldinii Casper and Steiger	*Pinguicula*	*P. leptoceras + allies*	J. Steiger 49 (JE)	AF531804	this study
Pinguicula vallisnerifolia Webb	*Pinguicula*	*P. vallisneriifolia + allies*	J. Steiger 23 (JE)	AF531805	this study
Pinguicula vulgaris L. (USA)	*Pinguicula*	*P. vulgaris + allies*	L. Legendre 1035 (NSW)	AF531807	this study
Pinguicula vulgaris L. (France)	*Pinguicula*	*P. vulgaris + allies*	L. Legendre 1034 (NSW)	AF531806	this study
Pinguicula emarginata Zamudio Ruiz and Rzedowski	*Temnoceras*	*P. moranensis + allies*	L. Legendre 1026 (NSW)	AF531785	this study
Pinguicula gracilis Zamudio Ruiz	*Temnoceras*	*P. filifolia + allies*	L. Legendre 1027 (NSW)	AF531790	this study
Genlisea aurea St. Hil.	*Genlisea*	*neotropical*	K. Müller 748	AF531814	this study
Genlisea hispidula Stapf	*Genlisea*	*African, group III*	K. Müller 727	AF531815	this study
Genlisea margaretae Hutchinson	*Genlisea*	*African, group I*	K. Müller 728	AF531816	this study
Genlisea roraimensis N. E. Brown	*Genlisea*	*neotropical*	K. Müller 729	AF531817	this study
Genlisea stapfii A. Chev.	*Genlisea*	*African, group II*	S. Porembski 3859 (ROST)	AF531818	this study
Genlisea uncinata P. Taylor and Fromm-Trinta	*Tayloria*	*Tayloria*	K. Müller 730	AF531819	this study
Utricularia blanchetii A. Dc.	*Aranella*	*Aranella*	K. Müller 704	AF531841	Müller and Borsch (subm.)
Utricularia parthenopipes P. Taylor	*Aranella*	*Aranella*	K. Müller 749	AF531842	Müller and Borsch (subm.)
Utricularia rigida Benj.	*Avesicarioides*	*Avesicarioides*	Porembski 3860 (ROST)	AF531838	Müller and Borsch (subm.)
Utricularia nana A. St. Hil. and Girard	*Benjaminia*	*Benjaminia*	K. Müller 750	AF531837	Müller and Borsch (subm.)
Utricularia livida E. Meyer	*Calpidisca*	*Calpidisca*	K. Müller 709	AF531833	Müller and Borsch (subm.)
Utricularia sandersonii Oliver	*Calpidisca*	*Calpidisca*	K. Müller 708	AF531847	Müller and Borsch (subm.)
Utricularia tridentata Sylvén	*Foliosa*	*Foliosa, Psyllosperma p.p.*	K. Müller 725	AF531825	Müller and Borsch (subm.)
Utricularia humboldtii Schomb.	*Iperua*	*Orchidioides, Iperua p.p.*	K. Müller 717	AF531836	Müller and Borsch (subm.)
Utricularia nephrophylla Benj.	*Iperua*	*Iperua p.p.*	K. Müller 720	AF531827	Müller and Borsch (subm.)
Utricularia reniformis A. St. Hil.	*Iperua*	*Iperua p.p.*	K. Müller 723	AF531828	Müller and Borsch (subm.)
Utricularia pubescens Sm.	*Lloydia*	*Lloydia*	S. Porembski 3852 (ROST)	AF531844	Müller and Borsch (subm.)
Utricularia foveolata Edgew.	*Oligocista*	*Oligocista*	S. Porembski 3850 (ROST)	AF531850	Müller and Borsch (subm.)
Utricularia spiralis Sm.	*Oligocista*	*Oligocista*	S. Porembski 3853 (ROST)	AF531851	Müller and Borsch (subm.)
Utricularia uliginosa Vahl	*Oligocista*	*Oligocista*	K. Müller 726	AF531849	Müller and Borsch (subm.)
Utricularia alpina Jacq.	*Orchidioides*	*Orchidioides, Iperua p.p.*	K. Müller 712	AF531822	Müller and Borsch (subm.)

continued →

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Table 1 continued

Species	Section/family	Name of clade in Fig. **3**	Voucher	GenBank accession number	Authors
Utricularia quelchii N. E. Br.	*Orchidioides*	*Orchidioides, Iperua p.p.*	K. Müller 722	AF531846	Müller and Borsch (subm.)
Utricularia dichotoma Labill.	*Pleiochasia*	*Pleiochasia*	K. Müller 714	AF531826	Müller and Borsch (subm.)
Utricularia multifida R. Br.	*Polypompholyx*	*Polypompholyx*	K. Müller 719	AF531848	Müller and Borsch (subm.)
Utricularia calycifida Benj.	*Psyllosperma*	*Foliosa, Psyllosperma p.p.*	K. Müller 705	AF531824	Müller and Borsch (subm.)
Utricularia hispida Lam.	*Psyllosperma*	*Psyllosperma*	K. Müller 716	AF531829	Müller and Borsch (subm.)
Utricularia longifolia Gardner	*Psyllosperma*	*Psyllosperma*	K. Müller 718	AF531834	Müller and Borsch (subm.)
Utricularia praelonga A. St. Hil. and Girard	*Psyllosperma*	*Psyllosperma*	K. Müller 720	AF531843	Müller and Borsch (subm.)
Utricularia flaccida A. Dc.	*Setiscapella*	*Setiscapella*	K. Müller 715	AF531830	Müller and Borsch (subm.)
Utricularia subulata L.	*Setiscapella*	*Setiscapella*	K. Müller 724	AF531821	Müller and Borsch (subm.)
Utricularia juncea Vahl	*Stomoisia*	*Stomoisia*	K. Müller 746	AF531832	Müller and Borsch (subm.)
Utricularia australis R. Br.	*Utricularia*	*Utricularia*	K. Müller 711	AF531823	Müller and Borsch (subm.)
Utricularia intermedia Hayne	*Utricularia*	*Utricularia*	T. Borsch, J. Wiersema, and B. H. Hellquist 3388	AF531839	Müller and Borsch (subm.)
Utricularia macrorhiza Leconte	*Utricularia*	*Utricularia*	K. Müller 703	AF531835	Müller and Borsch (subm.)
Utricularia olivacea Wright ex Griseb.	*Utricularia*	*Utricularia*	K. Müller 620	AF531840	Müller and Borsch (subm.)
Utricularia vulgaris L.	*Utricularia*	*Utricularia*	K. Müller 743	AF531831	Müller and Borsch (subm.)
Utricularia purpurea Walter	*Vesiculina*	*Vesiculina*	K. Müller 647	AF531845	Müller and Borsch (subm.)
Other families					
Dipteracanthus portellae (Hook f.) Boom	Acanthaceae	Acanthaceae	K. Müller 734	AF531773	this study
Thunbergia alata Boj. ex Sims	Acanthaceae	Acanthaceae	K. Müller 740	AF531811	this study
Avicennia germinans Moldenke	Avicenniaceae	Avicenniaceae	T. Borsch and V. Wilde 3094 (FR)	AF531771	this study
Campsis radicans Seem.	Bignoniaceae	Bignoniaceae	K. Müller 701	AF531775	this study
Kigelia africana Benth.	Bignoniaceae	Bignoniaceae	–	AF051988	Young et al. (1999)
Buddleja alternifolia Maxim.	Scrophulariaceae	Scrophulariaceae s.str. (incl. Myoporaceae, Buddlejaceae)	K. Müller 732	AF531772	this study
Byblis gigantea Lindl.	Byblidaceae	Veronicaceae p.p. + Byblidaceae	K. Müller 733	AF531774	this study
Streptocarpus bindseilii Eb. Fischer	Gesneriaceae	Gesneriaceae + Veronicaceae p.p.	E. Fischer 1006	AF531810	this study
Lamium maculatum L.	Lamiaceae	Lamiaceae	K. Müller 737	AF531780	this study
Ibicella lutea v. Eselt	Martyniaceae	Martyniaceae	K. Müller 735	AF531778	this study
Proboscidea louisianica (Mill.) Thell.	Martyniaceae	Martyniaceae	K. Müller 706	AF531809	this study
Myoporum montanum R. Br.	Scrophulariaceae	Scrophulariaceae s.str. (incl. Myoporaceae, Buddlejaceae)	K. Müller 738	AF531808	this study
Jasminum nudiflorum Lindl.	Oleaceae	Oleaceae	K. Müller 736	AF531779	this study
Uncarina grandidieri (Baill.) Stapf	Pedaliaceae	Pedaliaceae	K. Müller 707	AF531813	this study
Antirrhinum majus L.	Veronicaceae	Veronicaceae p.p. I	–	AF051978	Young et al. (1999)
Craterostigma hirsutum S. Moore	Scrophulariaceae	Veronicaceae p.p. + Byblidaceae	E. Fischer 9003	AF531776	this study
Gratiola officinalis L.	Scrophulariaceae	Veronicaceae p.p. II	K. Müller 710	AF531777	this study
Lathraea clandestina L.	Scrophulariaceae	Orobanchaceae	–	AF051989	Young et al. (1999)
Melampyrum sylvaticum L.	Scrophulariaceae	Orobanchaceae	–	AF051991	Young et al. (1999)
Orobanche caryophyllacea Sm.	Scrophulariaceae	Orobanchaceae	–	AF051992	Young et al. (1999)
Paulownia tomentosa Steud.	Scrophulariaceae	Paulowniaceae	–	AF051997	Young et al. (1999)
Pedicularis sylvatica L.	Scrophulariaceae	Orobanchaceae	K. Müller 744	AF531781	this study
Torenia vagans Roxb.	Scrophulariaceae	Veronicaceae p.p. + Byblidaceae	E. Fischer s.n.	AF531812	this study
Nicotiana tabacum L.	Solanaceae	Solanaceae	–	NC001879	Young et al. (1999)
Solanum tuberosum L.	Solanaceae	Solanaceae	–	Z11741	Du Jardin (1992)■
Verbena rigida Spreng.	Verbenaceae	Verbenaceae	K. Müller 742	AF531820	this study

trees per cycle. For Maximum Likelihood analyses (ML), the general time reversible model of nucleotide substitution (GTR+G+I) was used after initial testing of models with Modeltest (Posada and Crandall, 1998). Among site rate variation was assumed to follow a gamma distribution that was approximated by four rate categories, each represented by the mean. No molecular clock was enforced. The underlying nucleotide frequencies and the proportion of invariable sites were estimated from the data. For phylogeny reconstruction with ML, two matrices were derived from the data to reduce calculation time: one with a reduced sampling density in Lentibulariaceae (*Pinguicula vulgaris, Genlisea aurea, Utricularia*

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Table 2 Primers used in the present study

Name	Sequence (5′–3′)	Design
MG1[1]	AAC TAG TCG GAT GGA GTA GAT	Liang and Hilu (1996)
MG15[1]	ATC TGG GTT GCT AAC TCA ATG	Liang and Hilu (1996)
NymatK 480F[1]	CAT CTG GAA ATC TTG STT C	Borsch (2000)
psbAR[1]	CGC GTC TCT CTA AAA TTG CAG TCA T	Steele and Vilgalys (1994)
trnK 1280R	ATA AAA GCA AAC CCC TCT G	Müller et al. (subm.)
trnK 1280RA	ATA ACA GCA AAC CCT TCT G	Müller et al. (subm.)
trnK 1280RB	ATA CAA ACA AAA ACC TCT G	Müller et al. (subm.)
trnK 1580F[1]	TAC GAT TCT TTC TCA GCG	Müller et al. (subm.)
trnK 1580FC	TAC GAT TCT TGC TCA ACG	Müller et al. (subm.)
trnK 1590R	CTC GCT GAG AAA GAA TCG	Müller et al. (subm.)
trnK 1800R	AAA TGA AAG GAG TGG TTG C	Müller et al. (subm.)
trnK 2000F	CCT GAA TAA ATG GAA ATG	Müller et al. (subm.)
trnK 2050F	TTT TCC TTG TGG TTT CCT G	Müller et al. (subm.)
trnK 2450R	CTT TGT GTT TCC GAG CC	Müller et al. (subm.)
trnK 2600F	GTC GGA TTT GGT ATT TGG	Müller et al. (subm.)
trnK 2R[1]	AAC TAG TCG GAT GGA GTA G	Johnson and Soltis (1995)
trnK 3914Fdi[1]	GGG GTT GCT AAC TCA ACG G	Johnson and Soltis (1995)
trnK Le 1R[1]	ATA GAA ATA GAT TCG TTC	Müller et al. (subm.)
trnK Le 2F[1]	TGG TAC GGA GTC AAA KTC	Müller et al. (subm.)
trnK Le 3F[1]	ACC AAT CCT TTC ATT TAC G	Müller et al. (subm.)
trnK Le 4R[1]	TTC GCC TGA AAA TCC GTA ACC	Müller et al. (subm.)
trnK Le 7F[1]	GGG TTG CTA ACT CAA CGG TAG	Müller et al. (subm.)

[1] Used for amplification

vulgaris, data set partition I), and a second focusing on Lentibulariaceae only (using *Jasminum* as outgroup; data set partition II).

Additionally, Bayesian inference of phylogeny was used (BI) as implemented in the program MrBayes (Huelsenbeck and Ronquist, 2001), applying the same substitution model as above. The posterior probability was estimated by sampling trees from the posterior probability distribution, using the Metropolis-coupled Markov chain Monte Carlo algorithm. The temperature of the heated chain was set to 0.2 and chains were sampled every 10 generations. Four parallel chains were run for 100 000 generations for three iterations, each time starting with random trees. In all runs, the probabilities converged on a stable value after generation ~ 35 000. Therefore, calculations of the consensus tree and of the posterior probabilities of clades were based upon the trees sampled after this generation.

To compare the likelihood of alternative phylogenetic hypotheses with those found in the present study, Kishino-Hasegawa (KH) tests (Kishino and Hasegawa, 1989) were performed on trees with topologies enforced to match those of previously published trees.

Sequences of carnivorous and "proto-carnivorous" taxa in the Lamiales were subjected to relative rate tests using Maximum Likelihood estimates of substitutions per site (according to the model described above). These tests estimated standard errors via bootstrapping with help from the first author's program "Grate" and according to a procedure that is described in detail elsewhere (Müller et al., subm.). To compare substitutional rates of carnivores throughout the angiosperms, 292 additional *matK* sequences were added to the data set, representing most major lineages of angiosperms (obtained from GenBank). The program, GenBank accession numbers, alignments, and tables with rate differences and standard errors are available upon request and from http://www.botanik.uni-bonn.de/system/downloads/GRate.

Evolution of morphological characters

Morphological characters (Table **3**) were compiled in a character state matrix for subsequent analysis of character evolution (Table **4**). Characters of trap morphology and ultrastructure were originally obtained by scanning and transmission electron microscopy, as described in Reifenrath et al. (unpubl. data); other information was taken from published sources, as indicated in Table **3**. A difficulty for constructing the morphological data set was that actual data were not available for all species and that a compartmentalisation approach had to be followed (Mishler, 1994). Correct optimization actually requires determination of the ancestral character state in a compartment (see detailed discussion in Doyle et al., 2000). However, in the present situation it seemed appropriate to design compartments of different sizes (i.e., clade diversity) in order to have only one character state in a compartment. When trap structure was studied by SEM and TEM for only a single member out of a clade of closely related species, and no superficially deviating traps were observed in the other species, the same character states were assumed for these other species. A list of samples studied is available upon request. The matrix was used to optimize states of characters with MacClade (Maddison and Maddison, 1992), using the topology inferred by BI as

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Table 3 Morphological characters, states and codes. Emphasis was placed upon characters related to the carnivorous syndrome

0 – 23	***Vegetative morphology***
0 – 1	**Habit**
0	Life form: (0) aquatic plant; (1) epiphyte; (2) terrestrial. State assignments for *Utricularia* species based on Taylor (1989).
1	Morphology of photosynthetic organs in *Utricularia* (modified stems): (0) a solitary leaf-shaped organ with one central vein; (1) a complex branching system with either free filiform parts or parts fused, resulting in a complex bipartite venation system. State assignments based on Taylor (1989).
2 – 6	**Leaf morphology**
2	Leaf types on single plant: (0) epiascidiate leaves present; (1) not present. Bladder traps and eeltraps are considered homologous to leaves (Lloyd, 1942; Juniper, 1989).
3	Leaf tropism: (0) negative; (1) positive.
4	Position of leaves: (0) in rosettes; (1) on leaf-shaped modified stems or stolons (in *Utricularia*); (2) any other arrangement. Bladder traps and eel traps are considered homologous to leaves (Lloyd, 1942). State assignments for *Utricularia* species based on Taylor (1989).
5	Trichomes inside or at edge of traps ("Reusenhaare"): (0) absent; (1) present. Based on Reifenrath et al. (unpubl. data).
6	Leaf margins: (0) not connate; (1) partially connate; (2) completely connate. This also applies to leaves modified to traps.
7 – 14	**Trap morphology in *Utricularia* and *Genlisea***
7	Traps distally elongated into long helically twisted arms: (0) absent; (1) present.
8	Bladder trap with door: (0) absent; (1) present.
9	Trap appendages: (0) absent; (1) in pairs; (2) only one. Based on Reifenrath et al. (unpubl. data).
10	Form of trap appendages: (0) simple; (1) in rows, with glandular trichomes; (2) strongly branched (like antennae); (3) wing-shaped. Based on Reifenrath et al. (unpubl. data).
11	Position of trap mouth: (0) lateral; (1) basal; (2) terminal. Based on Reifenrath et al. (unpubl. data).
12	Trichomes on trap door (trigger hairs): (0) absent; (1) setaceous; (2) with glandular head. Based on Reifenrath et al. (unpubl. data).
13	Threshold: (0) missing (less than three cell layers); (1) consisting of three cell layers; (2) consisting of four or more cell layers. Based on Reifenrath et al. (unpubl. data).
14	Trap wall, number of cell layers: (0) two; (1) three or more. Based on Reifenrath et al. (unpubl. data).
15 – 21	**Gland morphology**
15	Digestive glands, number of underlying epidermal cells: (0) one; (1) two. Based on Lloyd (1942).
16	Digestive glands, attachment to vessels: (0) none; (1) present.
17	Mucilage glands, stem morphology: (0) situated on enlarged epidermal cell; (1) consisting of an elongated epidermal cell. Based on Lloyd (1942).
18	Glands with one head cell and no elongated stem cell on abaxial leaf surface (outer surface of traps): (0) absent; (1) present. Based on Lloyd (1942).
19	Glands on abaxial leaf surface, number: (0) few (sparsely covered); (1) many (densely covered). Based on Reifenrath et al. (unpubl. data).
20	Digestive glands in *Utricularia*: (0) only two-armed; (1) four- and two-armed. Based on Reifenrath et al. (unpubl. data) and Taylor (1989).
21	Mucilage glands: (0) losing turgor upon stimulation; (1) not so. Based on Lloyd (1942) and Legendre (2000).
22 – 23	**Root system**
22	Root system: (0) missing; (1) developed (weakly or strongly). Based on Lloyd (1942) and Juniper et al. (1989).
23	Primary root: (0) reduced immediately after germination; (1) fully developed. Based on Lloyd (1942) and Juniper et al. (1989).
24 – 28	***Reproductive morphology***
24	Calyx lobes: (0) two; (1) four; (2) five.
25	Flower buds in *Pinguicula*: (0) developed in spring after producing a first set of carnivorous leaves; (1) developed from winter resting bud before production of carnivorous leaves. Based on Legendre (2000) and personal observations.
26	Dehiscence of capsules in Lentibulariaceae: (0) spirally; (1) poricidal; (2) septicidal. Based on Taylor (1989) and Fromm-Trinta (1977).
27	Palatum: (0) absent; (1) present.
28	Spur: (0) absent; (1) present.

shown in Fig. **3**. This tree, inferred from an independent molecular data set, was selected because it is considered the best available hypothesis for phylogenetic relationships. We will consequently use the term "character optimization" to distinguish this approach from ancestral state reconstruction, where the respective characters are part of the matrix that is used to infer the tree. Since the selected characters are mostly those that were assumed to be part of the carnivorous syndrome, they are not representative for the whole plant. Therefore, the matrix was not used to calculate a phylogenetic tree solely based on morphology. For graphical purposes, the number of terminals representing a monophyletic group was reduced in Fig. **3**, and accordingly the branch lengths were optimised by ML with clades represented by one terminal after pruning taxa

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Table 4 Morphological matrix showing states for clades at the sectional level in *Utricularia and Pinguicula*, at the subgeneric level in *Genlisea,* at the generic level for the proto-carnivores, and states assigned to the remaining lineages of non-carnivorous Lamiales. See Table **3** for description of characters and states. A dash indicates absence of the respective character; ? = unknown character state

Taxa	Character number																											
	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28
Utricularia, sect. *Aranella*	2	0	0	1	1	1	2	0	1	1	0	0	1	1	0	0	1	1	1	?	?	1	0	0	0	–	2	1	1
Utricularia, sect. *Avesicarioides*	2	0	0	1	1	1	2	0	1	1	0	0	1	1	0	0	1	1	1	?	?	1	0	0	0	–	1	1	1
Utricularia, sect. *Benjaminiana*	2	0	0	1	1	1	2	0	1	0	0	1	1	1	0	0	1	1	1	?	?	1	0	0	0	–	2	1	1
Utricularia, sect. *Calpidisca*	2	0	0	1	1	1	2	0	1	1	1	2	1	1	0	0	1	1	1	0	1	1	0	0	0	–	2	1	1
Utricularia, sect. *Foliosa*	2	1	0	1	1	1	2	0	1	1	0	1	1	1	0	0	1	1	1	?	–	1	0	0	0	–	2	1	1
Utricularia, sect. *Orchidioides*	1	1	0	1	1	1	2	0	1	1	0	1	1	1	0	0	1	1	1	1	1	1	0	0	0	–	2	1	1
Utricularia, sect. *Iperua*	1	1	0	1	1	1	2	0	1	1	0	1	1	1	0	0	1	1	1	1	1	1	0	0	0	–	2	1	1
Utricularia, sect. *Lloydia*	2	0	0	1	1	1	2	0	1	1	1	2	1	1	0	0	1	1	1	?	?	1	0	0	0	–	2	1	1
Utricularia, sect. *Oligocista*	2	0	0	1	1	1	2	0	1	1	0	1	1	1	0	0	1	1	1	0	0	1	0	0	0	–	2	1	1
Utricularia, sect. *Pleiochasia*	2	0	0	1	0	1	2	0	1	1	3	0	0	2	0	0	1	1	1	0	1	1	0	0	0	–	2	1	1
Utricularia, sect. *Polypompholyx*	2	0	0	1	0	1	2	0	1	1	3	1	0	2	1	0	1	1	1	0	1	1	0	0	1	–	2	1	1
Utricularia, sect. *Psyllosperma*	2	1	0	1	1	1	2	0	1	1	0	1	1	1	0	0	1	1	1	1	1	1	0	0	0	–	2	1	1
Utricularia, sect. *Setiscapella*	2	0	0	1	1	1	2	0	1	1	0	0	1	1	0	0	1	1	1	0	1	1	0	0	0	–	1	1	1
Utricularia, sect. *Stomoisia*	2	0	0	1	1	1	2	0	1	0	0	0	0	1	0	0	1	1	1	0	1	1	0	0	0	–	2	1	1
Utricularia, sect. *Utricularia*	0	1	0	1	1	1	2	0	1	1	2	0	1	1	0	0	1	1	1	0	1	1	0	0	0	–	2	1	1
Utricularia, sect. *Vesiculina*	0	1	0	1	1	1	2	0	1	0	–	2	2	0	0	0	1	1	1	0	1	1	0	0	0	–	2	1	1
Genlisea, subg. *Tayloria*	2	–	0	1	0	1	1	1	0	–	–	–	–	–	–	0	1	–	1	–	–	1	0	0	2	–	0	1	1
Genlisea, subg. *Genlisea*	2	–	0	1	0	1	1	1	0	–	–	–	–	–	–	0	1	–	1	–	–	1	0	0	2	–	1	1	1
Pinguicula, sect. *Pinguicula*	2	–	1	0	0	0	0	0	0	–	–	–	–	–	–	0	1	0	0	–	–	0	1	0	2	0	2	0	1
Pinguicula, other sections	2	–	1	0	0	0	0	0	0	–	–	–	–	–	–	0	1	0	0	–	–	0	1	0	2	1	2	0	1
Ibicella	2	–	1	0	2	–	0	0	0	–	–	–	–	–	–	1	0	–	0	–	–	1	1	1	2	–	?	0	0
Byblis	2	–	1	0	2	–	0	0	0	–	–	–	–	–	–	1	0	0	0	–	–	1	1	1	2	–	?	0	0
Lamiales, other taxa	2	–	1	0	2	–	0	0	0	–	–	–	–	–	–	1	0	–	–	–	–	1	1	1	0, 1, 2	–	?	0, 1	0, 1
Solanaceae (outgroup)	2	–	1	0	2	–	0	0	0	–	–	–	–	–	–	1	0	–	–	–	–	1	1	1	2	–	?	0	0

from the tree (species inferred to the respective infrageneric clades are available from Table **1**). To facilitate the interpretation of tree topology, the length of non-terminal branches in the spine of the Lamiales tree up to the node showing Bignoniaceae, sister to Lentibulariaceae, were scaled to 300%. The branch leading to Lentibulariaceae was scaled to 50%.

Results and Discussion

Patterns of variability of the trnK *intron in Lamiales*

In the Lamiales, the *trnK* intron is 2490 bp (base pairs) long on average, ranging from 2435 to 2713 bp, from which 1491 to 1596 bp (mean 1531 bp) relate to the *matK* coding region (Fig. **1**). The 5′ part of the intron, upstream of *matK,* yielded an average of 702 (663 – 723) bp, whereas the shorter 3′ part provided 258 (166 – 490) bp. For all 89 taxa included, a complete reading frame was determined for *matK,* ranging from 497 to 532 codons. Indels occurred in both the *matK* coding region and in the noncoding intron parts, but were much more frequent in the coding region. Frameshift mutations are confined to the 3′ end of the gene, where they have little effect on the protein. In rare cases, deletions of four or five nucleotides were observed. Generally, these were found to be connected to particular sequence motifs (Müller and Borsch, subm.), in which the correct reading frame is re-established after two to three codons. In all data partitions (coding region and noncoding sections), the number of length mutations is much lower in *Pinguicula* than in *Utricularia* and *Genlisea.* A detailed analysis of length mutations and their phylogenetic utility, focusing on the genus *Utricularia,* will be published elsewhere. GC contents (33%) are comparable in all three genera of Lentibulariaceae, and do not significantly deviate from those observed in the non-carnivorous Lamiales. While the GC content of the 5′ noncoding part of the *trnK* intron is comparable to that of *matK* (approx. 34%), the smaller 3′ noncoding part is noticeably more AT-rich (30% GC). In the alignment, the *trnK* intron comprises 3058 characters, 1800 of which belong to the *matK* coding region. 1174 (38%) were constant, 1884 variable (62%), and 1321 (43%) were parsimony-informative. A significantly higher variability at 3rd positions in the *matK* coding region indicated the presence of purifying selection in the Lamiales, as previously observed in other *matK* data sets (Young and DePamphilis, 2000). These data and the general maintenance of a closed reading frame indicate the functionality of *matK* in Lamiales, despite strong differences in substitutional rates.

Phylogenetic relationships

A comparison of the trees inferred for the Lamiales using the three different approaches is presented in Fig. **2**. The topologies found with ML and BI are largely congruent, whereas the backbone revealed by MP is inconsistent. Parsimony searches yielded shortest trees of 6403 steps (CI 0.493, RI 0.767, RC 0.379). The strict consensus tree calculated for 89 taxa is given in Fig. **2** (left), where Lentibulariaceae are graphically simplified to three branches. The tree obtained by Bayesian inference is shown in the centre of Fig. **2**, again with a simplified illustration for Lentibulariaceae. The optimal tree found under the likelihood criterion for data set partition I (including only one species each of *Utricularia, Genlisea,* and *Pinguicula*) had a

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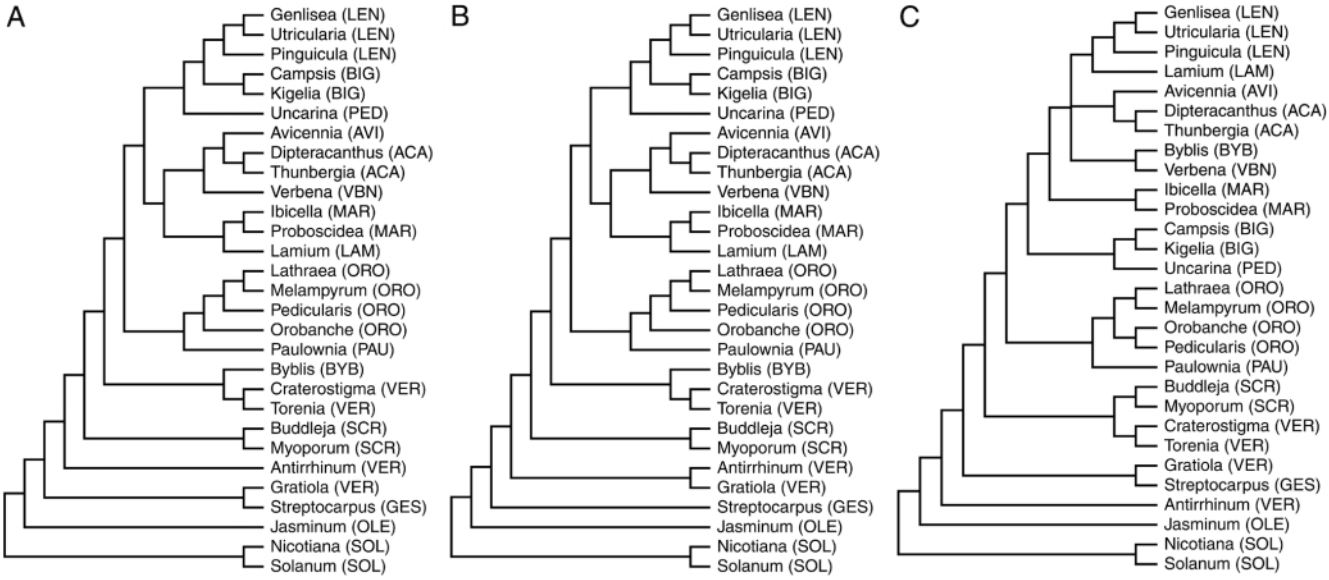

Fig. 2 Optimal trees found with Bayesian inference (**A**; calculated with the complete set of 89 taxa but Lentibulariaceae graphically reduced); maximum likelihood (**B**; –ln likelihood score 20399.088; only one species of *Utricularia, Genlisea* and *Pinguicula* was included in the analysis); and maximum parsimony (**C**; strict consensus calculated for 89 taxa, but Lentibulariaceae graphically reduced).

likelihood score of –ln 20399.088 and is depicted in Fig. **2** (right). In the analysis of Lentibulariaceae alone, with *Jasminum* as outgroup (data set partition II), the respective score was 23427.667.

The relationships inferred within the family Lentibulariaceae were fully congruent between the three methodological approaches, MP, ML, and BI (MP and BI calculated with unpartitioned data set). The Bayesian tree is shown as a phylogram (Fig. **3**) with posterior probabilities printed above the respective branch. For nodes with parsimony jackknife support (JK), values are shown in italics below (Fig. **3**; none of the nodes only found in MP but not in BI received JK values of > 50% so that differences in topology between MP and BI are inconsistent rather than incongruent). For graphical purposes, the number of terminals is reduced to 50% in this figure, and the remaining terminals are labelled with the name of the section or subgenus to which the covered species belong, following the classifications in Taylor (1989) for *Utricularia,* Fischer et al. (2000) for *Genlisea,* Casper (1966) and Legendre (2000) for *Pinguicula,* and Bremer et al. (2002) for families in the Lamiales. In case no name was available for a clade found in this study, another descriptive term was chosen (also listed in Table **1**). This largely applies to *Pinguicula,* since most previously described sections were found not to be monophyletic.

The monophyly of Lentibulariaceae is highly supported (100% JK, 100 PP). The same applies to the three genera, with *Pinguicula* appearing as sister group of a clade comprising *Genlisea* and *Utricularia*. The *Genlisea–Utricularia* subtree is completely resolved, and the majority of nodes have strong support (Fig. **2**). Such a high overall support is rarely observed in comparable data sets (e.g., Kron et al., 1999; Meimberg et al., 2001; Young et al., 1999). Contrary to the situation in *Pinguicula,* most of the currently accepted sections in *Genlisea* (Fischer et al., 2000; Fromm-Trinta, 1977) and *Utricularia* (Taylor, 1989) are supported by the tree inferred from our combined analysis of *matK* and *trnK* intron non-coding sequences (Fig. **3**). Congruent results were obtained by separate analyses of the non-coding and coding DNA data partitions (not shown). In *Pinguicula,* a split into two clades is apparent, one comprising northern temperate species (marked with "Sect. *Pinguicula*" in Fig. **3**) excluding *Pinguicula alpina,* and a second clade of predominantly Mexican distribution, including *P. alpina* as first branching species (Fig. **3**, character 25). This sister group rela-

Fig. 3 Phylogenetic relationships of carnivorous plants in the Lamiales as inferred by Bayesian analyses of the chloroplast *trnK* intron, including the *matK* gene. The number of terminals was reduced for graphical purposes to represent major clades only (see text). Posterior probabilities, showing the likelihood that the clade is correct, accompany the nodes. Parsimony jackknife support is also shown if > 50% (italics). Parsimony yielded no nodes with statistical support that are different from the Bayesian tree. Sketches of characteristic representatives accompany clades of carnivorous taxa (*Pinguicula* sect. *Pinguicula, Utricularia* sect. *Utricularia, Genlisea* subgen. *Genlisea*) and proto-carnivorous taxa (Martyniaceae: *Ibicella,* Byblidaceae: *Byblis*). Numbers in boxes indicate character state transformations of selected morphological characters (ACCTRAN optimisation). Character numbers refer to Table **3**. Open boxes represent reversals. When two different states arose from the same plesiomorphic state, the derived state is indicated after a slash. When a second transformation occurred, it is indicated after a dash. Illustrated transformations are as follows: 0/0 – aquatic plant; 0/1 – epiphyte; 1 – complex branching systems being photosynthetically active; 2 – presence of epiascidiate leaves; 3 – positive leaf tropism; 4 – leaves in rosettes; 4-1 – leaves on leaf-shaped modified stems; 7 – traps distally elongated in helically twisted arms; 8 – bladder traps with door present; 13 – threshold consisting of four or more cell layers; 15 – digestive glands with one underlying epidermal cell; 16 – digestive glands attached to vessels; 18 – glands with one head cell and no elongated stem cell on abaxial leaf surface present; 21 – Mucilage glands using turgor upon stimulation; 22 – root system missing; 23 – primary root reduced immediately after germination; 25 – flower buds developed before production of carnivorous leaves; 26 – capsule spirally dehiscent. ▶

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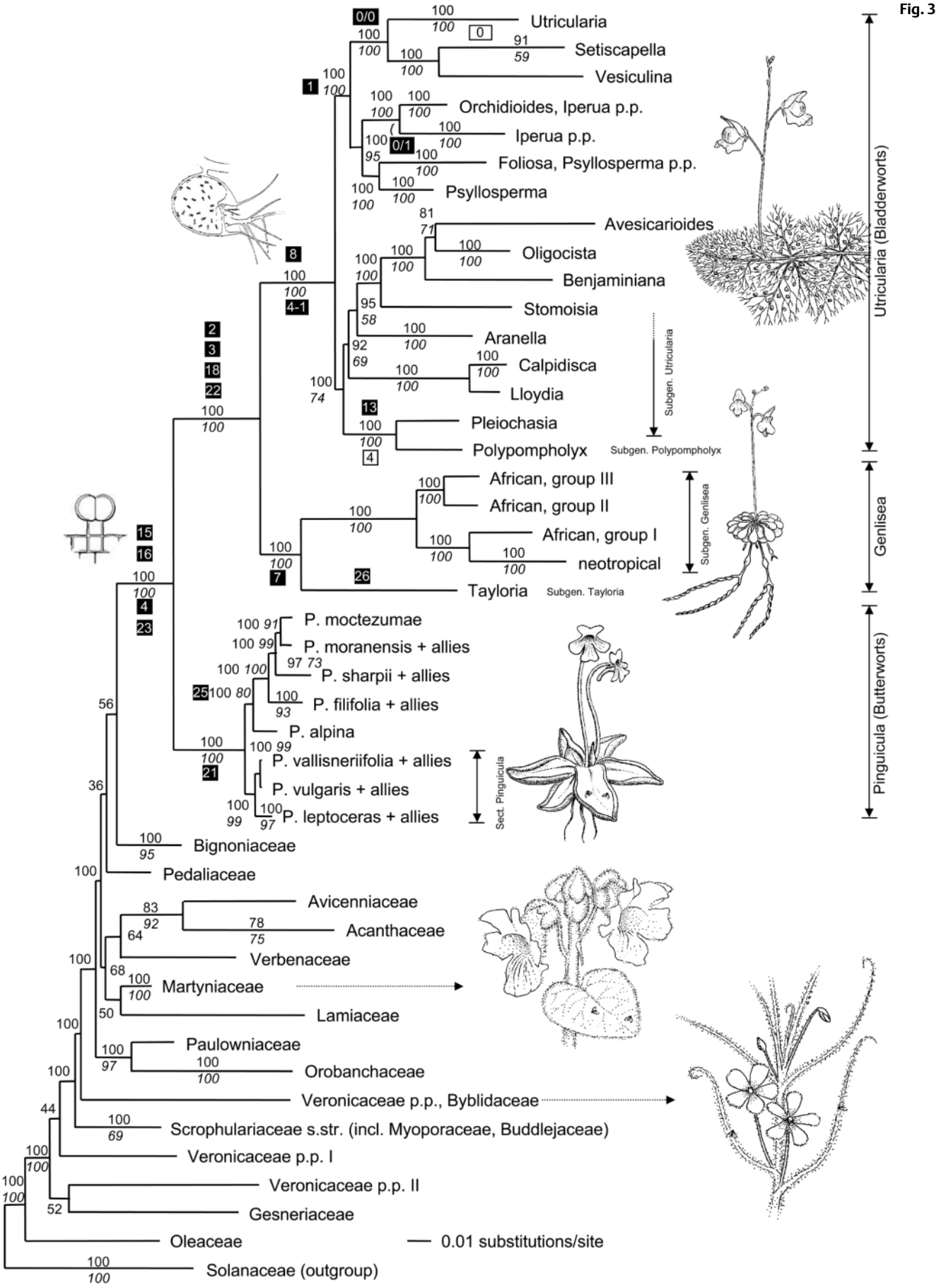
Fig. 3



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tionship of the temperate *P. alpina* to the Mexican clade is remarkable and may be supported by some vegetative characters, which will be discussed in detail elsewhere.

With respect to the interfamilial relationships in the Lamiales, there is some disagreement between trees inferred by the three different methods. BI provides evidence for Bignoniaceae as closest relative to Lentibulariaceae, though without convincing support (posterior probability 0.56). Values below 0.95 are here regarded as unsupported following theoretical considerations by Huelsenbeck et al. (2002) and Suzuki et al. (2002). ML agrees on this position of the Bignoniaceae, while under parsimony, Lamiaceae are sisters to Lentibulariaceae in the strict consensus, again without receiving jackknife support (Fig. **2**). Resolution and support for many other interfamilial relationships in the Lamiales are still rather low, similar to those observed in previous studies (Albach et al., 2001; Bremer et al., 2002; Hilu et al., 2003■; Olmstead et al., 2001; Olmstead and Reeves, 1995; Savolainen et al., 2000; Soltis et al., 2000; Young et al., 1999). All three methodologically different approaches (Fig. **2**) carried out for our data set agree on a most basal position of Oleaceae *(Jasminum),* which corresponds to the results of the studies just cited. Moreover, the non-monophyly of former Scrophulariaceae and Pedaliaceae is clearly supported here, in congruence with Olmstead et al. (2001), Olmstead and Reeves (1995), Young et al. (1999), as well as a clade of Orobanchaceae including hemi- and holoparasitic members of former Scrophulariaceae with Paulowniaceae as sister group. The relatively first branching position of Gesneriaceae and Plantaginaceae (= Veronicaceae) as depicted in our ML and BI trees (Fig. **2**) is in line with the findings of Bremer et al. (2002).

Although MP, ML, and BI differ in their exact placement of the "proto-carnivorous" Byblidaceae and Martyniaceae, all trees found in this study refuse a sister group relationship of either one to Lentibulariaceae (Figs. **2, 3**). Moreover, such a sister group relationship is also clearly rejected by KH tests ($p < 0.0001$), based on both likelihood and parsimony and using our combined *trnK* intron and *matK* sequence data. This implies that the carnivorous Lentibulariaceae and the "proto-carnivorous" Martyniaceae and Byblidaceae do not share an immediate common ancestor.

Previous phylogenetic studies including both carnivorous and "proto-carnivorous" Lamiales (Albert et al., 1992; Bremer et al., 2002) were compatible with scenarios depicting the evolution of carnivory as a stepwise process with the "proto-carnivores" as intermediates (Albert et al., 1992; Juniper, 1986; Juniper et al., 1989). However, neither of the underlying parsimony trees had good support for the respective nodes, and putatively long branches in *Genlisea* and *Utricularia* were omitted by not sampling them. Those studies with more extensive sampling in the Lamiales did not include Lentibulariaceae or "proto-carnivores" and exclusively used MP (Albach et al., 2001; Olmstead et al., 2001; Olmstead and Reeves, 1995; Oxelman et al., 1999; Savolainen et al., 2000; Soltis et al., 2000; Young et al., 1999). During this work it was observed that, under parsimony, the branching patterns inferred among families of Lamiales dramatically depend on the sampling, both in terms of density and selection of taxa. This may be explained by highly deviating branch lengths among the different lineages within the Lamiales, and varying effects of long branch attraction, to which MP is more sensitive than ML and BI (e.g., Huelsenbeck, 1995). According to our *trnK* intron data with dense sampling in the carnivorous lineages which have the longest branch lengths, and using a range of optimality criteria for phylogenetic inference, the hypothesis of a single origin of the carnivorous syndrome including the "proto-carnivores" is significantly less parsimonious (MP, KH) and likely (ML, Bayesian, KH) than alternatives that assume at least two independent origins of the syndrome in the Lamiales.

Origin of carnivory in the Lamiales

The carnivorous syndrome (Juniper, 1986) is constituted by an array of different characters, among which leaf architecture, root system, and gland morphology are central. Tracing the appearance of individual character states on the phylogenetic tree (Fig. **3**) provides insights into the differentiation process of the syndrome. Stalked glands are found in most families of the Lamiales (Fig. **3** shows a sketch of a particular stalked gland type). They are usually secretory and protect against insect predation. In particular, the production of mucilage that glues insects to the leaf is regarded as a defence against small herbivores and has been called "defensive trapping" (Juniper, 1986; Juniper et al., 1989). Moreover, leaf surface proteinase activity has been detected in several Lamiales families (Spomer, 1999). The secreted enzymes digest accidentally trapped insects as well as other organic material.

Evidence has been provided for the ability of glands to quite easily shift their function from secretion to absorption (Ziegler and Lüttge, 1959). Parsimony reconstruction of character evolution suggests that the common ancestor of the Lentibulariaceae has acquired absorptive glands that were attached to tracheid elements and consisted of one epidermal cell supporting an endodermoid cell and glandular head cells occurring in multiples of two (Fig. **3**, characters 15 ad 16; see schematic sketch). Glands of this type can absorb amino acids, nucleotides, and organic substances released during the decomposition of insects and other organic matter by digestive enzymes (Dixon et al., 1980; Lüttge, 1983). With help of the activities of these glands, insects and other small organisms became prey, providing additional macronutrients. The energetically expensive reduction of nitrogen oxides (Marschner, 1995) was supplemented by direct uptake of N organic molecules (e.g., amino acids) and also ammonium from the prey. These compounds were previously unavailable through the roots since free macromolecules are first consumed by microorganisms in a natural environment and since roots cannot secrete proteinases to allow direct access (Marschner, 1995). Such a supply is likely to have increased the fitness of a plant growing in low-nutrient habitats, and may be regarded as a crucial factor during the evolution of true carnivory in the Lamiales.

Mapping traits onto the phylogeny further suggests that the radiation of all lineages in Lentibulariaceae started with terrestrials, including the immediate common ancestor of the family. Both the submerged aquatic and epiphytic life forms in *Utricularia* represent derived conditions (Fig. **3**, character 0). This common ancestor of the Lentibulariaceae is reconstructed to have possessed a basal rosette composed of flat leaves and a primary root, reduced soon after germination (Fig. **3**, characters 4 and 23). A rosette was later lost with the adaptation to aquatic habitats in a terminal lineage of *Utricularia.* While in

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the *Pinguicula* lineage adventitious roots were maintained, roots were completely lost in the ancestor of *Utricularia* and *Genlisea* (Fig. **3**, character 22), possibly because root functions were taken over by the leaves or shoots.

Inverse tropism and closing the leaves abaxially (yielding epiascidiate leaves) are central progressions in the *Utricularia–Genlisea* lineage (Fig. **3**, characters 2 and 3). Although the traps in extant *Utricularia* and *Genlisea* appear to have little in common with the flypaper traps in *Pinguicula,* studies of their ontogeny revealed that they are modified epiascidiate leaves (Lloyd, 1942; Rutishauser and Sattler, 1989). Also, mutants with epiascidiate pitchers instead of flat leaves are known from various angiosperms (Juniper, 1986) including *Pinguicula,* suggesting that only small alterations in the genotype are required for this step. Inverse tropism is not uncommon in plants, as exemplified by pendent epiphytic life forms. The selection pressure behind reversing tropism most likely stems from the rich prey spectrum that became available when mutations caused some leaves of the rosette to closely contact and finally enter the soil. In this new environment, catches could include protists and other microscopic organisms (Barthlott et al., 1998; Seine et al., 2002), which are more numerous and constitute more biomass than insects above the ground, making catches more frequent and more reliable. The access to new resources resulted in increased fitness and led to further specializations that increased trapping efficiency. In *Genlisea,* traps developed with two helically twisted arms (Fig. **3**, character 7, thumbnail sketch to the right) that increase the surface area exposed to water, while *Utricularia* evolved traps with a door and a doorstep for tight closure and a trigger system to open on demand (Fig. **3**, character 8, top left sketch). Contrary to *Genlisea,* most *Utricularia* species arrange their traps in a scattered manner (Taylor, 1989), thereby maximizing the portion of a habitat to be exploited (cf. thumbnail sketch of *Utricularia* at the top of Fig. **3**).

Correlation of high substitutional rates with nutritional specialization

The morphological and physiological changes in the Lentibulariaceae lineage were accompanied by an increase in evolutionary speed on the molecular level. Substitutional rates in the *trnK* intron, including the *matK* gene, are considerably higher in *Genlisea* and *Utricularia* than in *Pinguicula,* and are also higher than in any other angiosperm so far examined (Fig. **4**). A more detailed analysis of relative rates, comparing other nutritionally specialized angiosperms and groups within carnivorous genera, including information from other genes and other genomes (Müller et al., submitted), points toward gene-independent rate acceleration in *Genlisea* and *Utricularia*. Longer branches in *Utricularia* compared to *Pinguicula* species have also been reported from additional genomic regions (Jobson and Albert, 2002).

The extreme rate acceleration found in *Genlisea* and *Utricularia* parallels the extreme specialization in the carnivorous syndrome in these two genera, in a sense that trapping microfauna opened up a continuous resource resulting in certain levels of reliance. Carnivory is known to supply intact biosynthetic building blocks, such as amino acids, peptides, and nucleotides (Dixon et al., 1980; Lüttge, 1983). As these are key intermediates in various heavily branched metabolic pathways, an external supply can be expected to lower selective pressures on metabolic pathways related to those building blocks, resulting in relaxed functional constraints on the structure of involved enzymes. High mutational rates as observed in *matK* and also other genomic regions of *Utricularia* and *Genlisea* may therefore be linked to relaxed functional constraints, caused by the uptake of larger building blocks.

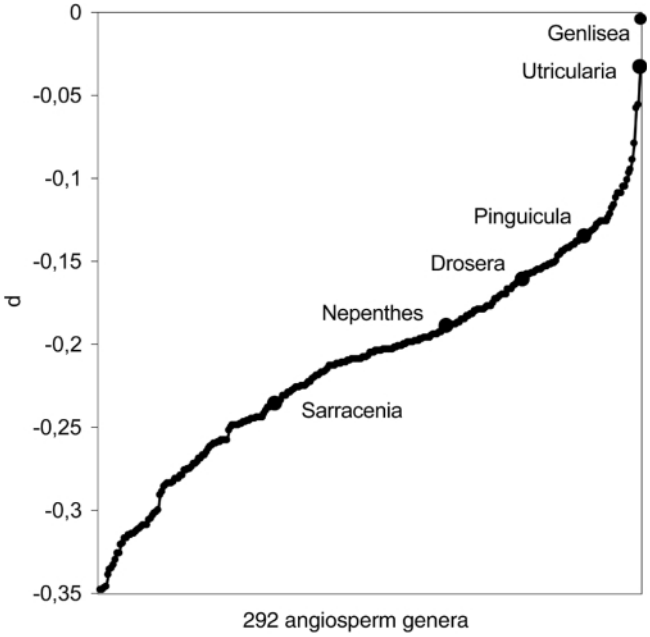

Fig. 4 Relative substitutional rates of *matK* in 292 angiosperm genera representing 200 families and most orders, compared to *Genlisea* (outgroup: *Amborella* + Nymphaeales). X axis: taxa, y axis: $d = K(Genlisea, \text{outgroup}) - K(\text{taxa, outgroup})$; with $K(i,j)$ = maximum likelihood estimate (GTR + G + I model) of substitutions per site between taxa *i* and *j*.

In other lineages of carnivorous plants, such as Sarraceniales and Nepenthales as well as in *Pinguicula,* rates are not substantially increased (Fig. **4**; Müller et al., submitted). Contrary to *Utricularia* and *Genlisea,* these carnivores benefit from unpredictable and rather infrequent catches of animal prey (Juniper et al., 1989). *Utricularia* and *Genlisea,* however, are characterized by a strongly differing trap environment and prey spectrum that includes soil and water microfauna (Barthlott et al., 1998; Seine et al., 2002), which helps to guarantee more constant prey availability. Higher dependency of *Utricularia* on carnivory than of other lineages is further indicated by ontogeny. Traps are the first structures to arise after the very minute cotyledons. These cotyledons are not photosynthetically active and plants have to rely on catching prey as a source of organic compounds at this stage (Lloyd, 1942). Experiments have revealed that feeding peptone and glutamine to *Utricularia* species improved their growth over that obtained with pure mineral nutrition (Pringsheim and Pringsheim, 1967). Elevated mutational rates have also been demonstrated for parasitic plants (Nickrent et al., 1998). Parasites, like carnivores, tend to exploit alternative nutrient sources compared to most green plants, and accelerated rates might also be associated with relaxed functional constraints on certain parts of their genomes (Nickrent et al., 1998).

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Previous hypotheses explaining rate heterogeneity include the generation time effect (Wu and Li, 1985), differences in metabolic rate (Martin and Palumbi, 1993), and varying DNA repair efficiencies (Britten, 1986), yet none of these appears sufficient to totally explain the rate patterns presented here (Müller et al., subm.). Positive correlations of species diversity and rates have been reported in a number of angiosperms (Barraclough and Savolainen, 2001) and were also used to explain longer branches found in *Utricularia* versus *Pinguicula* (Jobson and Albert, 2002). However, in the case of the Lentibulariaceae, a correlation of rates and species numbers is not obvious, and is even contradicted by the fact that *Utricularia* has slightly lower rates than *Genlisea* while comprising about ten times as many species. Moreover, rates in *Utricularia* and *Genlisea* clearly exceed those of other angiosperm clades, regardless of their diversity, indicating that in Lentibulariaceae, the effects of reproductive isolation expressed in the speciation rate may be of less importance among the factor controlling rates.

Conclusion

The integration of results from molecular phylogenetics, morphology and ecology as presented here is used to gain insights into mechanisms involved in molecular and structural evolution that can be of general relevance. Lentibulariaceae as an extremely specialized lineage of plants have proven to be useful for hypothesizing new mechanisms involved in controlling DNA mutational rates, which now can be further tested. The present results show that the analysis of extreme systems with strong shifts in biochemical or morphological characters will offer valuable additions to the analysis of "traditional" model organisms like *Arabidopsis,* thereby underscoring that considering organismal diversity may lead to insights into biological processes of broad relevance. It may be worth mentioning that mechanisms like the function of the Golgi apparatus (Schnepf, 1961) have also been unravelled using carnivorous plants. Carnivory at first glance may appear as one of the miracles in plant evolution, but the results presented here indicate that in essence remarkable phenotypic progressions may be explained by rather simple evolutionary steps. This is underlined by the fact that central components of the carnivorous syndrome have evolved several times independently in "proto-carnivores" and cranivores within a single lineage of closely-related angiosperms (Lamiales).

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