

Morphology and nrITS Phylogeny of the Genus *Pinguicula* L. (Lentibulariaceae), with Special Attention to Embryo Evolution

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Abstract: The genus *Pinguicula* (Lentibulariaceae) is unusual within the dicot order Lamiales because of the occurrence of both embryos with two cotyledons and those with just one cotyledon. In order to elucidate the infrageneric relationships and the evolutionary history of the embryo, we analysed (1) the internal transcribed spacers ITS1 and ITS2 of the nuclear ribosomal DNA (nrITS) of 29 Old and New World taxa of *Pinguicula*, and (2) the morphological and anatomical characters of the seeds. We suggest that the cotyledon number and spermoderm structure were quite unstable in the evolution of *Pinguicula*. Although basal nodes of the nrITS tree are sensitive to taxon sampling, all tree topologies found in this study imply homoplasy in the cotyledon number.

Key words: Carnivorous plants, Lentibulariaceae, *Pinguicula*, nrITS phylogeny, embryo evolution.

Introduction

The family Lentibulariaceae is characterised by a great variability in spermoderm and embryo structures, including cotyledon number. In this respect, the family is probably unique in the Lamiales. The genus *Pinguicula* containing about 100 species, is unusual because some of its species possess embryos with two cotyledons while others have only one cotyledon (e.g., Dickson, 1869; Netolitzky, 1926; Haccius and Hartl-Baude, 1957; Studnička, 1986; Nikiticheva, 1987; Degtjareva et al., 2004). It is difficult to comprehend the origin of an embryo with a single cotyledon in *Pinguicula* and other dicots such as some Umbelliferae, Ranunculaceae, and Primulaceae (see Haccius, 1952, 1953, 1954; Haccius and Hartl-Baude, 1957; Eames, 1961; Titova, 1998, 2000). Treviranus (1839, 1848) considered that the so-called single cotyledon in *Pinguicula* is in fact the first leaf (eophyll) and he suggested that cotyledons are lacking. In contrast, Goebel (1932) assumed that a fusion of the two cotyledons had taken place. The embryo development of two species of *Pinguicula* has been studied in detail by Haccius

and Hartl-Baude (1957). They concluded that monocotyl results from complete loss of the second cotyledon. Similar conclusions were drawn by Velenovský (1907) and by Crété (1956). Titova (2000) concluded that a suppression of one cotyledon (that would result in its complete loss) and the fusion between two cotyledons are two intimately associated phenomena in *Pinguicula* as well as in some other dicot taxa. Theories similar to those mentioned above were proposed to explain the origin of a single cotyledon in monocots (e.g., Arber, 1925; Meyer, 1960; Kudryashov, 1964; Takhtajan, 1991; Titova and Batygina, 1996; Burger, 1998; Titova, 2003, see also Lodkina, 1988; Tzvelev, 2000).

Degtjareva et al. (2004) published a comparative study of seed and embryo morphology of 19 species of *Pinguicula* from all subgenera established by Casper (1966) in the last world-wide revision of the genus.

The aim of the study by Degtjareva et al. (2004) was a better understanding of the pattern of the cotyledon number variation, since only a few species of the genus had been investigated before in this respect. Significant differences in spermoderm anatomy and ultrasculpture have also been discovered among the studied species. Spermoderm and embryo morphology has not yet been compared to any molecular phylogenetic data.

In this paper we present a molecular phylogeny of *Pinguicula* based on the nuclear ribosomal ITS (nrITS) sequences of Schmidt (1998) and Degtjareva (2002). We also compare seed and embryo structure in *Pinguicula* with our nrITS phylogeny of the genus as well as with the chloroplast phylogeny of Cieslak et al. (2005) in order to elucidate the evolutionary history of seed and embryo structure in *Pinguicula*.

Materials and Methods

Complete sequences of the nrITS region were obtained for 49 accessions representing 29 taxa of the genus *Pinguicula*, i.e., 25 species, some of which are represented by different subspecies, varieties, and by different localities (Table 1; taxonomy after Casper, 1966). We used two species of the genus *Utricularia* (*U. intermedia* and *U. reniformis*) as outgroup species. The voucher information of all samples investigated is shown in Table 1. The voucher specimens are deposited in Jena (JE),

Table 1 Genbank accession numbers and sources of nrITS sequences used in this study. The letters in parentheses after the taxon names allow us to understand the origin of the taxa shown in the phylogenetic tree of Fig. 2

Species	GenBank number	Voucher data (extracted tissue)
<i>Pinguicula agnata</i> Casper	DQ441602	Botanical Garden, Jena, Germany, <i>Casper P0052</i> (leaves)
<i>P. alpina</i> L. (A)	DQ222969	Romania, Transylvanian Alps, W Sinaia, <i>Steiger S 41</i> (seeds)
<i>P. alpina</i> L. (B)	DQ438092	Italy, Seiser Alm near Bozen, <i>Schmidt s.n.</i> (seeds)
<i>P. alpina</i> L. (C)	DQ438100	Switzerland, Canton Bern, between Habkern and Grünenberg-Pass, 2001, <i>Steiger P0017</i> (leaves)
<i>P. balcanica</i> Casper	DQ222954	Bulgaria, S of Sofia, <i>Steiger S 30</i> (seeds)
<i>P. bohemia</i> Krajina	DQ441597	Czech Republic, SE of Ceska Lipa, E of village Jestrebi, Baronsky rybnic, <i>Steiger P0028</i> (leaves)
<i>P. caerulea</i> Walter	DQ222963	USA, Georgia, SW of Folkston, <i>Schlauer P0043</i> (leaves)
<i>P. corsica</i> M. Bernard et Gren. ex Gren. et Godr. (A)	DQ222955	France, Corsica, Lac de Melo, <i>Steiger S 18</i> (seeds)
<i>P. corsica</i> M. Bernard et Gren. ex Gren. et Godr. (B)	DQ438098	France, Corsica, Lac de Melo, <i>Steiger P0018</i> (leaves)
<i>P. corsica</i> M. Bernard et Gren. ex Gren. et Godr. (C)	DQ438090	France, Corsica, Lac de Melo, <i>Steiger s.n.</i> (leaves)
<i>P. crystallina</i> Sibth. ex Sibth. et Smith (A)	DQ222965	Greece, Cyprus, <i>Steiger S 36</i> (seeds)
<i>P. crystallina</i> Sibth. ex Sibth. et Smith (B)	DQ438082	Greece, Cyprus, Kakopetria, Ayios Nicolaos, <i>Steiger s.n.</i> (leaves)
<i>P. dertosensis</i> (Canig.) Schlauer	DQ441598	Spain, Prov. Tarragona, Sierra de Caro/Sierra de Fortalesa, <i>Steiger P0033</i> (leaves)
<i>P. fiorii</i> Tammaro et Pace	DQ222952	Italy, Maiella, Bocca di Valle W of Guardiarelle, <i>Steiger S 27</i> (seeds)
<i>P. grandiflora</i> Lam. subsp. <i>grandiflora</i> f. <i>grandiflora</i> (A)	DQ222958	France, Dept. Hautes Pyrenées, <i>Steiger S 12</i> (seeds)
<i>P. grandiflora</i> Lam. subsp. <i>grandiflora</i> f. <i>grandiflora</i> (B)	DQ438099	France, Dept. Ain, <i>Steiger P0007a</i> (leaves)
<i>P. grandiflora</i> Lam. subsp. <i>grandiflora</i> f. <i>grandiflora</i> (C)	DQ438091	Spain, Picos de Europa, Rio Cares, <i>Schmidt s.n.</i> (leaves)
<i>P. grandiflora</i> subsp. <i>grandiflora</i> f. <i>pallida</i> (Gaudin) Casper (A)	DQ222957	France, Dept. Ain, <i>Steiger S 9</i> (seeds)
<i>P. grandiflora</i> subsp. <i>grandiflora</i> f. <i>pallida</i> (Gaudin) Casper (B)	DQ438097	France, Dept. Ain, <i>Steiger P0007b</i> (leaves)
<i>P. grandiflora</i> subsp. <i>rosea</i> (Mutel) Casper (A)	DQ222956	France, Dept. Isère, between Concelin and Sollières NE of Grenoble, <i>Steiger S 11</i> (seeds)
<i>P. grandiflora</i> subsp. <i>rosea</i> (Mutel) Casper (B)	DQ438081	France, Dept. Isère, Col du Granier, <i>Steiger P0008</i> (leaves)
<i>P. hirtiflora</i> Ten. (A)	DQ222966	Italy, near Salerno, <i>Steiger S 34</i> (seeds)
<i>P. hirtiflora</i> Ten. (B)	DQ438083	Greece, Thessalia, Mount Olympus, <i>Debbert s.n.</i> (leaves)
<i>P. leptoceras</i> Rchb.	DQ222947	Italy, Col di Tende, <i>Steiger S 15</i> (seeds)
<i>P. longifolia</i> Ramond ex DC. subsp. <i>longifolia</i> (A)	DQ222959	France, Dept. Hautes Pyrenées, <i>Steiger S 20</i> (seeds)
<i>P. longifolia</i> Ramond ex DC. subsp. <i>longifolia</i> (B)	DQ438089	France, Central Pyrenées, <i>Steiger s.n.</i> (leaves)
<i>P. longifolia</i> subsp. <i>caussensis</i> Casper (A)	DQ222948	France, Dept. Lozère, Gorge du Tarn, <i>Steiger S 21</i> (seeds)
<i>P. longifolia</i> subsp. <i>caussensis</i> Casper (B)	DQ438095	France, Dept. Lozère, Gorge du Tarn, <i>Steiger P0020</i> (leaves)
<i>P. longifolia</i> subsp. <i>caussensis</i> Casper (C)	DQ438088	France, Dept. Lozère, Gorge du Tarn, <i>Steiger s.n.</i> (leaves)
<i>P. longifolia</i> subsp. <i>reichenbachiana</i> (Schindler) Casper (A)	DQ222950	France, Alp. Maritimes, Roya Valley, <i>Steiger S 22</i> (seeds)
<i>P. longifolia</i> subsp. <i>reichenbachiana</i> (Schindler) Casper (B)	DQ438094	France, Alp. Maritimes, Roya Valley, <i>Steiger P0019</i> (leaves)
<i>P. longifolia</i> subsp. <i>reichenbachiana</i> (Schindler) Casper (C)	DQ438087	France, Roya Valley, <i>Steiger s.n.</i> (leaves)
<i>P. lusitanica</i> L.	DQ222960	Spain, Rio de la Miel near Algeciras, <i>Schmidt s.n.</i> (seeds)
<i>P. lutea</i> Walter	DQ222962	USA, Alabama, S. Elsauer, <i>Schlauer P0039</i> (leaves)
<i>P. macroceras</i> Link subsp. <i>nortensis</i> J. Steiger ex J. Steiger et H. Rondeau	DQ222951	USA, northernmost California, del Norte County, <i>Steiger S 28</i> (seeds)
<i>P. moranensis</i> Kunth	DQ222967	Botanical Garden, Jena, Germany, <i>Casper P0006</i> (leaves)
<i>P. mundi</i> Zamora, Jamilena, Ruiz-Rejon et Blanca	DQ441599	Spain, Prov. Albacete, near border to Prov. Jaen, <i>Steiger P0022</i> (leaves)
<i>P. planifolia</i> A.W. Champ	DQ441601	USA, Florida, Apalachicola Forest near Sumatra, <i>Schlauer P0040</i> (leaves)
<i>P. poldinii</i> Casper et Steiger	DQ441600	Italy, Friaül, Campone, <i>Casper P0049</i> (leaves)
<i>P. primuliflora</i> C. E. Wood et Godfrey	DQ222964	USA, Florida, S Crestvien, <i>Schlauer P0042</i> (leaves)
<i>P. vallisneriifolia</i> Webb (A)	DQ222953	Spain, Sierra de Cazorla, Cueva de la Magdalena, <i>Steiger S 26</i> (seeds)
<i>P. vallisneriifolia</i> Webb (B)	DQ438084	Spain, Sierra de Cazorla, Cueva de la Magdalena, <i>Schmidt s.n.</i> (leaves)
<i>P. variegata</i> Turcz.	DQ222968	Russia, Khabarovskiy kray, near Okhotsk, <i>Steiger S 29</i> (seeds)

continued →

Table 1 continued

Species	GenBank number	Voucher data (extracted tissue)
<i>P. villosa</i> L. (A)	DQ222961	Cultivated material, <i>Steiger S 40</i> (seeds)
<i>P. villosa</i> L. (B)	DQ438096	Norway, Soer-Troendelag, <i>Steiger P0036</i> (leaves)
<i>P. villosa</i> L. (C)	DQ438085	Sweden, Abisko, <i>Günter s.n.</i> (leaves)
<i>P. vulgaris</i> L. (A)	DQ222949	Iceland, <i>Steiger S 38</i> (seeds)
<i>P. vulgaris</i> L. (B)	DQ438086	Germany, Altenberga near Jena, <i>Schmidt s.n.</i> (leaves)
<i>P. vulgaris</i> L. (C)	DQ438093	Switzerland, Canton Bern, <i>Steiger P0038a</i> (leaves)
<i>Utricularia intermedia</i> Hayne	DQ225109	European Russia, Tver' Prov., <i>Notov et al. s.n.</i> (MW)
<i>U. reniformis</i> A. St. Hil.	DQ225108	Brazil, Organ Mountains, <i>Nerz</i> (leaves)

except for the voucher of *Utricularia intermedia*, which is deposited in Moscow (MW). Total DNA was isolated from seed and leaf tissue using the CTAB method (Doyle and Doyle, 1987). PCR was performed using universal primers (ITS-P1 and ITS-P4 [White et al., 1990] synthesised by MWG-Biotech). Both spacer regions were sequenced entirely on both strands. All obtained sequences were aligned manually using the SED program of the VOSTORG package (Zharkikh et al., 1991). The complete alignment is available on request from the corresponding author.

Maximum parsimony and Bayesian analyses were performed for the nrITS datasets. The GTR+I+ Γ model of nucleotide substitutions was selected by the AIC (Akaike Information Criterion) in Modeltest 3.7 (Posada and Crandall, 1998). Bayesian inference of phylogeny was explored using the MrBayes program (version 3.1; Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003). 5 000 000 generations were performed and trees from the first 4 500 000 generations were discarded. The number of generations to be discarded was determined using a convergence diagnostic. Two heated chains were used. Four runs were performed for each of four datasets (see below) to avoid suboptimal plateaus. The Bayesian consensus trees are thus derived from 2 000 000 trees (500 000 trees from each of the four runs). Equally weighted parsimony analyses including a heuristic search with random addition of taxa (100 replicates) were conducted using PAUP* (version 4.0b8; Swofford, 2003). Bootstrap values (Felsenstein, 1985) were calculated from 1000 replicate analyses with TBR branch swapping and random addition of taxa. In the parsimony analyses all gaps were treated as missing data.

Four nrITS datasets (i.e., sub-sets of the total of 43 aligned sequences) have been analysed. Dataset #1 includes one nrITS sequence for each taxon studied by Degtjareva et al. (2004) with respect to seed and embryo structure. Dataset #2 also includes species studied previously with respect to seed and embryo structure, but, in contrast to dataset #1, most taxa are represented here by two sequences obtained from different individuals (Table 1). Dataset #2 was generated to test for the possibility of infraspecific variation of nrITS sequences. If the variation is present, analysis of dataset #2 should provide understanding of its significance regarding robustness of phylogenetic reconstructions in *Pinguicula* based on nrITS. Dataset #3 was generated to test the impact of taxon sampling on the nrITS tree topology. Dataset #3 includes all sequences of dataset #1 plus sequences of six more *Pinguicula* species for which

we do not have seed structure data. Dataset #4 was generated to test the impact of possible reticulate evolution for nrITS tree topology in *Pinguicula*. This dataset differs from dataset #3 by omitting *P. crystallina*, *P. hirtiflora*, and *P. variegata*. *Pinguicula crystallina* and *P. hirtiflora* are closely related and, based on intermediate morphological features, it was suggested that they form a hybrid complex derived from ancient crossing of *P. lusitanica* (sect. *Isoloba*) and a member of the section *Pinguicula* (such as *P. corsica* and *P. leptoceras*) (Mikeladse, 1996; Schmidt, 1998). Thus, a hybridisation event between members of different subgenera recognised by Casper (1966) is assumed.

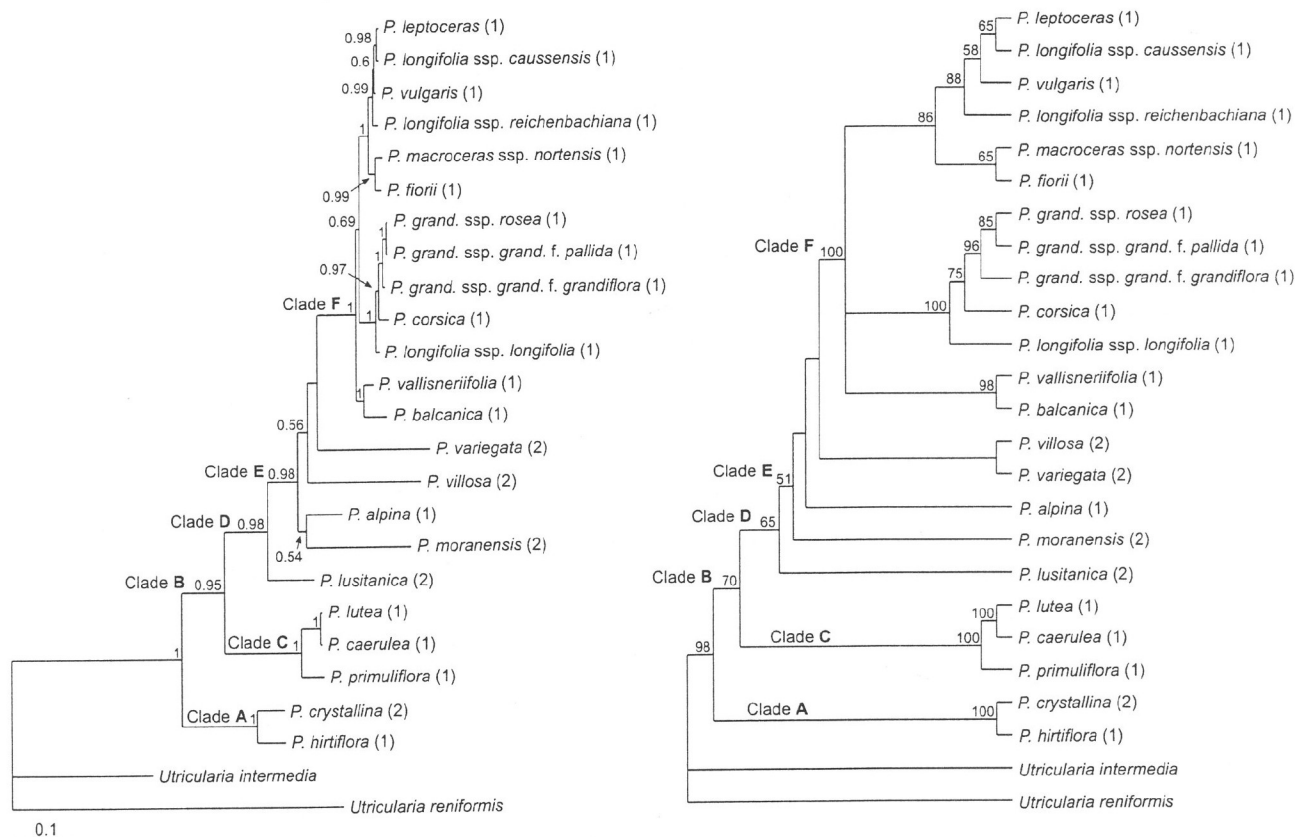
Results and Discussion

Characterisation of nrITS sequences

In the 41 obtained nrITS sequences of *Pinguicula* (datasets #1, 2, and 3), the length of the nrITS1 region ranges from 242 to 266 bp, that of 5.8S from 162 (one of three sequences of *P. longifolia* subsp. *reichenbachiana*) to 163 bp (all other sequences of *Pinguicula*), and that of the nrITS2 region from 204 to 222 bp. The total length of the nrITS region (ITS1+5.8S+ITS2) varies from 618 to 645 bp. The nrITS region of *U. reniformis* (674 bp) is longer than that of *Pinguicula*, while the nrITS of *U. intermedia* (509 bp) is much shorter than that of *Pinguicula*. The GC content is relatively stable in 5.8S (53–57%), but much more variable in ITS1 and ITS2 regions. The GC content in ITS1 is lowest in *Utricularia reniformis* (49%). Among the *Pinguicula* species the lowest GC content was found in *P. hirtiflora* (55–56%) and the highest in *Pinguicula alpina* (74%). In ITS2 the GC content is lowest in *Utricularia reniformis* (48%). Among the *Pinguicula* species the lowest GC content in ITS2 was found in *P. hirtiflora* (56–57%) and the highest in *Pinguicula agnata* (75%). The nrITS region is GC-rich in many angiosperms (e.g., Hershkovitz and Zimmer, 1996). It has been suggested that a low GC content allows identification of sequences as pseudogenes (e.g., Álvarez and Wendel, 2003). We do not believe that our sequences are pseudogenes because: (1) in our sequences the GC content was much more stable in the 5.8S region than in the nrITS regions; (2) when we produced two or three sequences from different localities of the same taxon, they were quite similar or identical in their GC content; (3) our preliminary data on modelling secondary structures of the ITS2 region show normal secondary structures in the taxa investigated. Helices I, II, and III which are similar to those in other angiosperms (e.g., Hershkovitz and Zimmer, 1996; Wolf et al., 2005) were revealed in all taxa; and (4) sequences of the

Table 2 Main features of datasets used for phylogenetic analyses

Dataset #	Number of <i>Pinguicula</i> sequences	Number of parsimony-informative sites		Number of parsimony-uninformative sites		Number of constant sites	
		Including outgroup	Ingroup only	Including outgroup	Ingroup only	Including outgroup	Ingroup only
1	23	259	224	132	98	241	310
2	43	298	275	102	59	232	298
3	29	273	236	133	110	226	286
4	26	240	198	142	104	250	330

**Fig. 1** Phylogenetic trees inferred from analyses of dataset # 1. Left: Bayesian tree with posterior probabilities of nodes. Right: strict consensus tree of the three most parsimonious trees (length = 893 steps,

CI = 0.66, RI = 0.69) with bootstrap support of nodes (1000 replicates). The major clades of *Pinguicula* are indicated. The cotyledon number is given in parentheses after taxon names.

same taxon group together in our phylogenetic trees (see below).

The alignment of all 49 sequences of *Pinguicula* and two sequences of *Utricularia* results in a matrix of 632 nucleotide positions after excluding 248 ambiguous positions that could not be unambiguously aligned due to common length polymorphism.

Molecular phylogenetic analyses

Main features of datasets used in phylogenetic analyses (number of parsimony-informative, parsimony-uninformative, and constant sites) are summarised in Table 2.

Analyses of dataset # 1

The matrix contained 23 *Pinguicula* sequences representing 23 taxa. Phylogenetic trees inferred from Bayesian and parsimony analyses were similar in their general topology (Fig. 1). *Pinguicula crystallina* and *P. hirtiflora* (both Mediterranean species of the section *Cardiophyllum*, subgen. *Isoloba*) formed a clade (clade A, PP – posterior probability – of 1.00, BS – bootstrap support – of 100%) which was the sister to all other sampled *Pinguicula* species (clade B, PP 0.95, BS 70%). Clade B comprised the clades C and D. Clade C (PP 1.00, BS 100%) includes three North American species of the section *Isoloba* (subgen. *Isoloba*): *P. caerulea*, *P. lutea*, and *P. primuliflora*. Only in the Bayesian analysis (PP 0.98, BS 65%) was clade D well support-

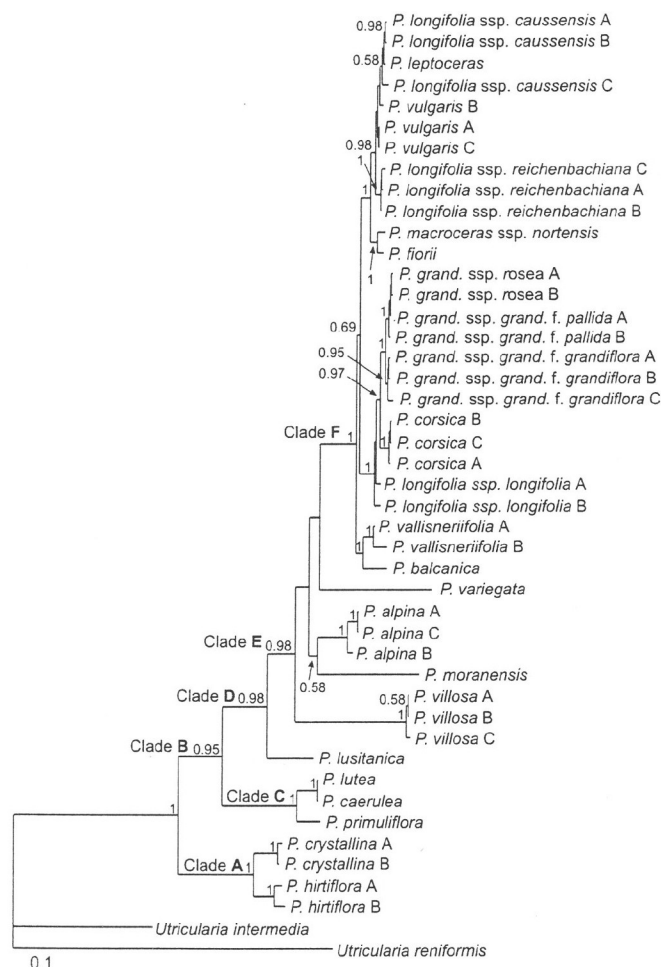
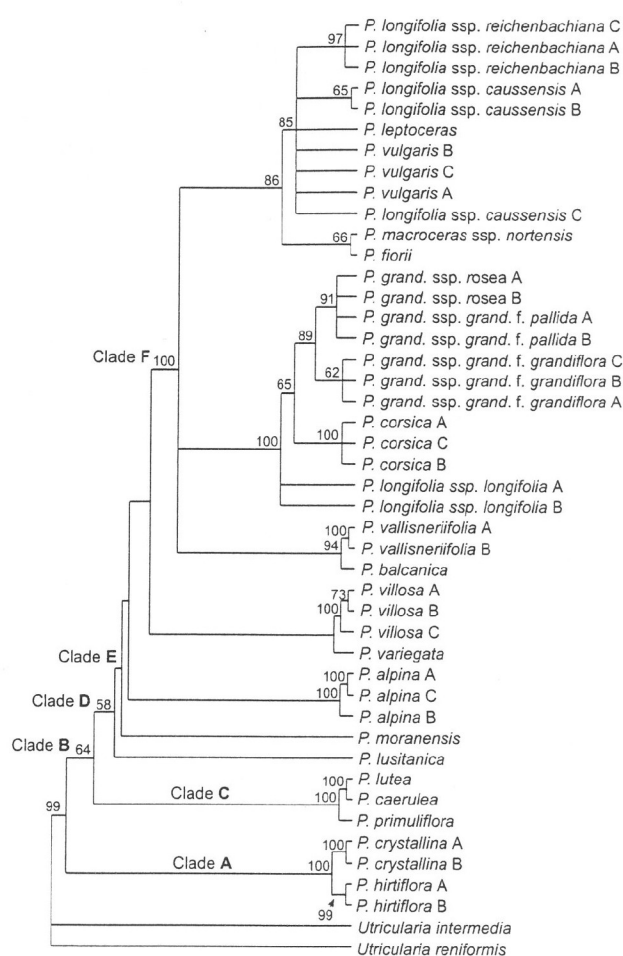


Fig. 2 Phylogenetic trees inferred from analyses of dataset #2. Left: Bayesian tree with posterior probabilities of nodes. Right: strict consensus tree of the 108 most parsimonious trees (length = 940 steps, CI = 0.64, RI = 0.81) with bootstrap support of nodes (1000 replicates).



The major clades of *Pinguicula* are indicated. The letters after taxon names correspond to different sources of material as outlined in Table 1.

ed. *Pinguicula lusitanica*, the only European species of section *Isoloba*, takes the basal position in clade D. It is sister to clade E, which was well supported only in the Bayesian analysis (PP 0.98, BS 51%). Clade E includes members of subgenera *Temnoceras* (*P. alpina* and *P. variegata*) and *Pinguicula* (*P. villosa* of sect. *Nana*, *P. moranensis* of sect. *Orcheosanthus*, and a large clade F comprising members of section *Pinguicula*). Although clade F was well supported (PP 1.00, BS 100%), relationships with other species of clade E were not clear. *Pinguicula villosa* was sister to *P. variegata* in the strict consensus tree of the most parsimonious trees, but the BS of this grouping was below 50%; *P. alpina* was sister to *P. moranensis* in the Bayesian tree, but with a PP of 0.54 only.

Although all members of the section *Pinguicula* have relatively similar nrITS sequences (pairwise comparisons of percentage sequence divergence vary between 0% and 9.5%), phylogenetic analyses revealed three clades within the section. The first clade includes *P. balcanica* and *P. vallisneriifolia* being a sister group to the rest of the section in the Bayesian analysis, but this relationship received a relatively low posterior probability (0.69) and does not persist in parsimony trees. The second

clade includes *P. corsica*, *P. grandiflora*, and *P. longifolia* subsp. *longifolia*. The third clade comprises *P. fiorii*, *P. leptoceras*, *P. macroceras*, *P. vulgaris*, and two subspecies of *P. longifolia* which do not tend to group together.

Analyses of dataset #2

The matrix contained 43 *Pinguicula* sequences representing 23 taxa. If a single taxon was represented from more than one locality, its terminal clades grouped together in the phylogenetic trees or rarely formed polytomies with other terminals or clades (Fig. 2).

Polytomies are formed by the lack of sequence divergence between closely related taxa. For example, two sequences of *P. grandiflora* subsp. *rosea* were almost identical as well as with two sequences of *P. grandiflora* subsp. *grandiflora* f. *pallida*. Therefore all four terminals form a polytomy.

The overall topology of trees inferred from maximum parsimony analyses of dataset #2 was the same as that found in analyses of dataset #1. Bayesian inferred consensus trees showed

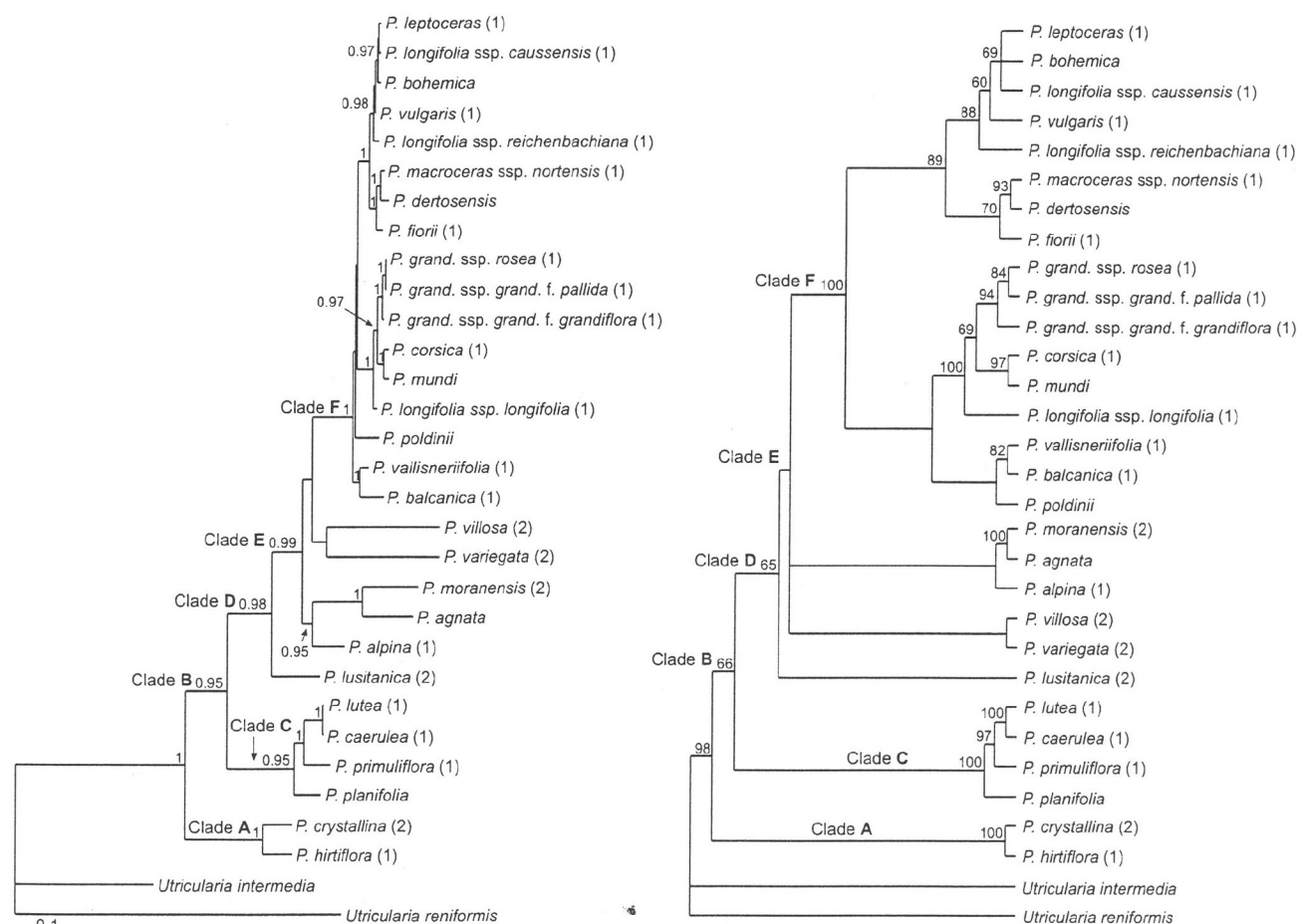


Fig. 3 Phylogenetic trees inferred from analyses of dataset #3. Left: Bayesian tree with posterior probabilities of nodes. Right: strict consensus tree of the two most parsimonious trees (length = 987 steps,

CI = 0.63, RI = 0.71) with bootstrap support of nodes (1000 replicates). Major clades of *Pinguicula* are indicated. The cotyledon number is given in parentheses after taxon names.

some differences in basal branching within clade E. In the Bayesian tree derived from dataset #1, *Pinguicula alpina* + *P. moranensis* were basal within clade E, whereas *P. villosa* was basal in clade E in the Bayesian tree derived from dataset #2. However, posterior probabilities of both topological alternatives were low.

Analyses of dataset #3

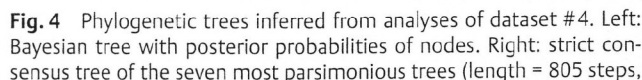
The matrix contained 29 *Pinguicula* sequences representing 29 taxa. Adding more species of *Pinguicula* resulted in only minor changes to the overall tree topology (Fig. 3). Bayesian trees inferred from analyses of datasets #1 and #3 were alike. The only topological difference was that *P. villosa* grouped with *P. variegata* in the tree derived from dataset #3. However, this grouping received only a PP of 0.4. An alternative grouping of these species in the tree derived from the dataset #1 also received a low PP. A grouping of *P. villosa* with *P. variegata* was found in the maximum parsimony analysis of dataset #1 as well as in maximum parsimony analysis of dataset #2.

Adding the Mexican *Pinguicula agnata* to the dataset, caused it to group with another Mexican species, *P. moranensis* (PP 1.00, BS 100%). *Pinguicula alpina* was the sister to *P. moranensis* + *P.*

agnata, with a PP of 0.95. In the analysis of dataset #1 (where *P. agnata* was absent), *P. alpina* grouped with *P. moranensis*, but the PP of this grouping was only 0.54. A similar, but much less pronounced effect was obtained in the maximum parsimony analysis. When *P. agnata* was absent (dataset #1), *P. alpina* did not group with *P. moranensis*. When *P. agnata* is added, a clade of *P. alpina* and *P. agnata* + *P. moranensis* was present in the strict consensus tree but received a BS of less than 50%.

Analyses of dataset #4

The matrix contained 26 *Pinguicula* sequences representing 26 taxa. Excluding the putative inter-sectional hybrid species (*Pinguicula variegata*, *P. crystallina*, and *P. hirtiflora*) slightly changed the placement of the putative parents of *P. variegata* (*P. alpina* and *P. villosa*). In the Bayesian tree inferred from dataset #3 (Fig. 3), *P. villosa* + *P. variegata* formed a poorly supported clade as sister to the section *Pinguicula* (i.e., clade F). When *P. variegata* was excluded from this dataset (Fig. 4), *P. villosa* was basal in clade E. Omitting *P. crystallina* and *P. hirtiflora* caused no change in Bayesian tree topology (Fig. 4). *Pinguicula villosa* was basal in the strict consensus tree of the most parsimonious trees derived from analysis of dataset #4 (Fig. 4), but this topology had a BS of only 69%. That this placement of *P.*



CI = 0.70, RI = 0.74) with bootstrap support of nodes (1000 replicates). The major clades of *Pinguicula* are indicated. The cotyledon number is given in parentheses after taxon names.

Phylogenetic significance of nrITS sequence data in *Pinguicula*

Despite the outgroup problem and many other problems associated with the use of nrITS data (reviewed in Álvarez and Wendel, 2003; Bailey et al., 2003), we believe that our study contributes useful information on infrageneric relationships in *Pinguicula*. In terms of rooting problems, our nrITS data are less equivocal than the chloroplast DNA data obtained by Cieslak et al. (2005). In that study the tropical growth-type clade appeared to be sister to the rest of the genus, but the monophyly of the remaining species of the genus was only weakly supported (BS 63% or < 50%, PP<0.5). It is possible to combine *trnK* intron and nrITS sequences in the hope of producing a robust phylogeny. However, such combining of nuclear and chloroplast data is not reasonable in groups with possible reticulate evolution (see below).

We believe that the use of four different nrITS datasets in the present study, which provided similar (although sometimes not identical) tree topologies, allows us to discuss systematic aspects and character evolution patterns in *Pinguicula*.

Comparison with previous molecular phylogenetic studies of *Pinguicula* and with morphology-based classification

Our nrITS data are congruent with data obtained from the same DNA region in a preliminary study by Shimai and Kondo (2004), although they explored a slightly different set of taxa

and an independent set of vouchers (they also have not yet published a complete analysis of their data). Shimai and Kondo (2004) also found the basal position of *Pinguicula crystallina* + *P. hirtiflora*, although they used *Gentlisea* instead of *Utricularia* as outgroup.

The chloroplast DNA phylogeny of *Pinguicula* published by Cieslak et al. (2005) shares many features with our nrITS phylogeny. In particular, the monophyly of section *Pinguicula* is strongly supported by both studies. Seed anatomy data also suggest naturalness of this group (Degtjareva et al., 2004). Although sequence divergence was very high for the *trnK* intron as well as for nrITS at the family level, the divergence was very low at the level of section *Pinguicula* in both DNA markers. It seems that nrITS is slightly more informative than the *trnK* intron at the level of section *Pinguicula*. In contrast to our study, Cieslak et al. (2005) found a polytomy of seven taxa of section *Pinguicula*. In the well resolved part of the section *Pinguicula* clade, Cieslak et al. (2005) found grouping of *P. leptoceras*, *P. longifolia* subsp. *reichenbachiana*, and *P. poldinii*, (BS 96%, PP 1.00). The placement of these three taxa was entirely different in our phylogeny. This may indicate an extensive reticulate evolution within section *Pinguicula*.

Many aspects of our tree topology are congruent with the morphology-based classification of *Pinguicula* by Casper (1996). Examples are the monophyly of section *Pinguicula* and the monophyly of subsection *Primuliformis* (this includes SE North American *P. caerulea*, *P. lutea*, *P. planifolia*, *P. primuliflora* in our study). In other aspects, however, our trees are not congruent with the morphology-based classification. Each of the three subgenera recognised by Casper was not monophyletic in our study. Most studied members of the subgen. *Isoloba* form a basal grade in most of our trees, while *P. agnata* groups with *P. moranensis* of subgen. *Pinguicula*. The placement of *P. agnata* has strong BS and PP in our analyses and this is also congruent with the chloroplast phylogeny of Cieslak et al. (2005), as well as with biogeography (Mexican distribution) and with some aspects of floral morphology (e.g., the petals are not emarginate). *Pinguicula alpina* and *P. variegata* represent the subgenus *Temnoceras* in our study. Although the placement of these species is not equivocal in our analyses, they never grouped together. Cieslak et al. (2005) also did not find these two species grouping together. Furthermore, none of the seed characters analysed by Degtjareva et al. (2004) suggest a sister relationship between *P. alpina* and *P. variegata*.

An important conclusion that could be drawn from nrITS phylogeny is the paraphyly of *Pinguicula longifolia* s.l. since its subspecies *P. longifolia* subsp. *longifolia*, *P. longifolia* subsp. *causensis*, and *P. longifolia* subsp. *reichenbachiana* fall in different clades (see also Schmidt, 1998). This paraphyly was also obtained by Cieslak et al. (2005) in the analysis of chloroplast DNA sequences. Seed-morphological data also suggest that the three subspecies of *P. longifolia* should be treated as separate species (Degtjareva et al., 2004).

The close relationship of *Pinguicula crystallina* and *P. hirtiflora* is confirmed by our study. In our opinion, the isolated and basal position of section *Cardiophyllum* can be explained by a possible former hybridisation event. Mikeladse (1996) refers to the ambivalent character of these taxa and, on the basis of her morphological and chromosomal investigations, she assumed

a hybridisation of ancestors with basic chromosome numbers of $x = 6$ and $x = 8$. The basic number $x = 6$ would originate from an *Isoloba* taxon, e.g., *P. lusitanica*. An allotetraploid number of $2n = 28$ found by Mikeladse (1996) could be a result of hybridisation of diploid taxa followed by a polyploidisation. Apart from *P. crystallina* and *P. hirtiflora* the tropical homophyllous growth form is only found in *P. lusitanica* in Europe. The bilabiate flower structure, however, occurs in sections *Pinguicula* and *Micranthus*. Comparing flower morphology, Mikeladse concluded that the second possible parent species had to be a representative of section *Pinguicula* with characters similar to *P. corsica* and *P. leptoceras*. There is no inconsistency with our results of a hybrid origin for section *Cardiophyllum*. Assuming an equalisation of the sequences of both parent species over a long time after the hybridisation, a new sequence could have developed, which is dissimilar to the initial sequences.

Evolution of seed coat and embryo characters in *Pinguicula*

Seed and embryo characters have been described in detail by Degtjareva et al. (2004; see also Fig. 5). These characters are shown in Fig. 6, along with the phylogenetic trees based on nrITS and chloroplast DNA data. Assuming some flux in tree topologies and, especially, difficulties in morphological comparisons with the large outgroup genus *Utricularia*, we decided not to give parsimonious character optimisations. The seeds of *Utricularia* are as diverse as in *Pinguicula* (e.g., Taylor, 1994) and it is not yet possible to recognise ancestral seed character states in *Utricularia*.

Seeds of *Pinguicula* (Fig. 5) develop from anatropous, unitegmic, and tenuinucellar ovules and the nucellus decays during the early stages of embryo sac development (Haccius and Hartl-Baude, 1957; Poddubnaya-Arnoldi, 1982). Seeds are only 0.4–1.0 mm and rarely (*P. variegata*) up to 1.5 mm long. Four studied *Pinguicula* species have especially small seeds (less than 0.6 mm long). Three of them form a well-supported North American clade (*P. caerulea*, *P. lutea*, *P. primuliflora*). A fourth species, *P. lusitanica*, forms a subsequent clade in our trees. It is unclear whether very small seeds are derived within *Pinguicula*, since most other members of the family Lentibulariaceae (in the genera *Utricularia* and *Gentlisea*) produce very small seeds but larger seeds are common among more distantly related outgroups. When we exclude putative hybrid taxa such as *P. crystallina* and *P. hirtiflora* from our analysis, small-seeded species form a basal grade in *Pinguicula*. In the phylogeny of Cieslak et al. (2005) all small-seeded species fall into an apparently basal tropical growth-type clade. Most species of *Pinguicula* have ellipsoidal seeds. Only four species among those studied here have spindly seeds. This condition appears to be derived, with at least three independent origins in the genus (in *P. fiorii*, *P. vallisneriifolia* and possibly in an ancestor of *P. alpina* and *P. moranensis* – or in each of four species independently).

All studied species have seeds with an appendage at the micropylar end (Figs. 5A,B). The length of the micropylar appendage in *Pinguicula* varies considerably, and the evolution of this character is obviously homoplastic. Some species have seeds with a second, chalazal appendage (Fig. 5A; a more pronounced chalazal appendage is illustrated in Degtjareva et al., 2004, Fig. 3). Although this character is quite homoplastic, in most species of section *Pinguicula* the chalazal appendage is absent.

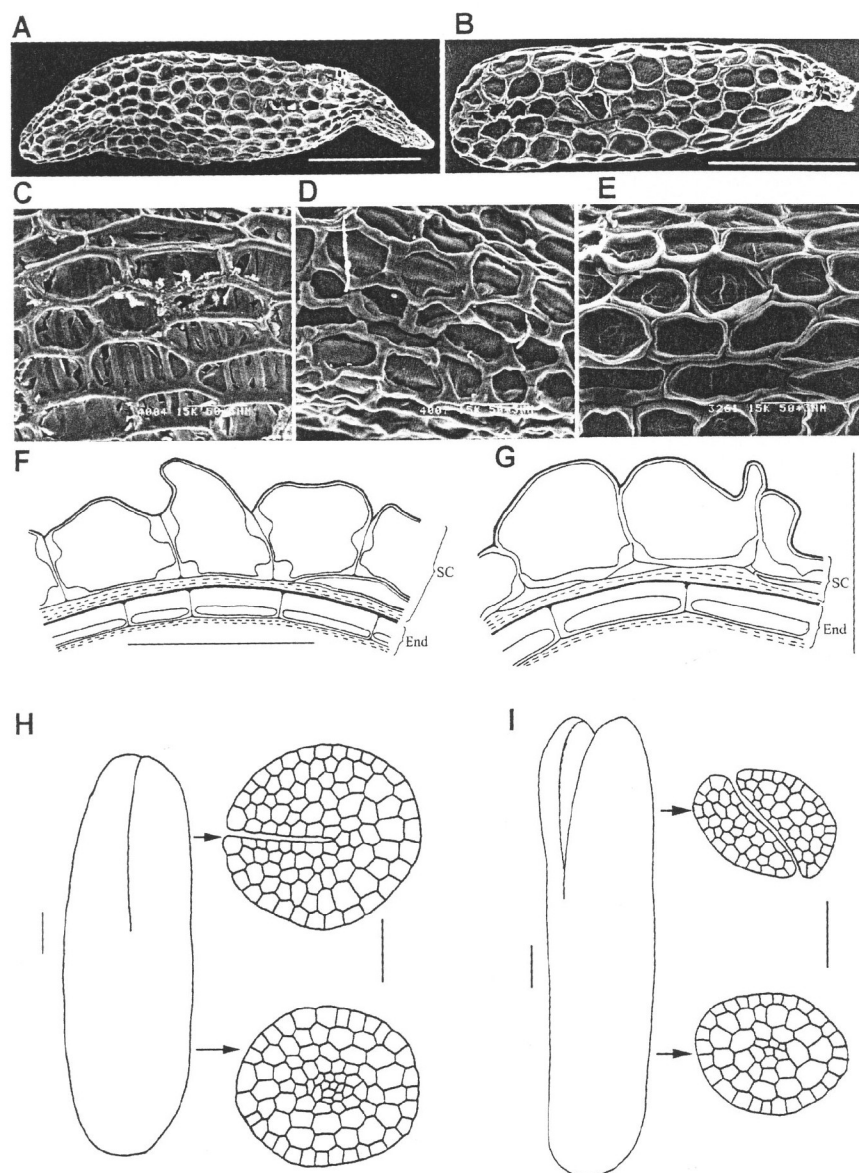


Fig. 5 Seed and embryo morphology in *Pinguicula*. (A, B) General view of the seeds (SEM), scales = 300 μ m. A *P. crystallina*, long micropylar appendage (right side). B *P. grandiflora* f. *pallida*, short micropylar appendage (right side). (C–E) Details of the seed coat surface ultrasculpture in the middle part of the seeds (SEM), scales = 50 μ m. C *P. lutea*, presence of cracks in the outer wall of exotesta cells and absence of furrows between the outer parts of anticlinal walls of adjacent cells. D *P. hirtiflora*, absence of cracks in the outer wall of exotesta cells and absence of furrows between the outer parts of the anticlinal walls of adjacent cells. E *P. leptoceras*, absence of cracks in the outer wall of exotesta cells and presence of furrows between the outer parts of the anticlinal walls of adjacent cells. (F, G) Details of cross-sections of three seed coats (SC) and endosperm (End), scales = 100 μ m. F *P. longifolia* subsp. *longifolia*, anticlinal walls of the exotesta cells are thickened in their interior and outer parts and not thickened in the middle part. G *P. grandiflora* f. *pallida*, anticlinal walls of the exotesta cells are thickened in their interior part only. (H, I) Embryo structure, scales = 100 μ m. H *P. vallisneriifolia*, one cotyledon. I *P. moranensis*, two cotyledons.

Zamora et al. (1996), Mikeladse (1996), and Degtjareva et al. (2004) have described a wide diversity of seed coat surface ultrasculptures in the genus *Pinguicula*. Among these characters, details of contacts between adjacent exotesta cells appear to be the most informative character. In all studied members of the section *Pinguicula* as well as in *P. lusitanica*, a furrow divides outer parts of anticlinal walls of adjacent cells. In other examined species, outer parts of anticlinal walls of adjacent exotesta cells are almost completely united. The presence of a furrow is clearly resolved as an apomorphic feature using our phylogenetic framework.

The seed coat of *Pinguicula* consists of exotesta, mesotesta, and endotesta. The exotesta always has large, non-obiterated cells. The outer walls of the exotesta cells are always thin. *Pinguicula caerulea* and *P. lutea* differ from the rest of the species investigated here in the presence of cracks in the outer walls of the exotesta cells in mature seeds (Fig. 5C). These two species are closest relatives in our molecular trees. Seeds of these two spe-

cies (and some others that are not included in the present study) share not only the cracks but also some other important seed characters, plus a similar geographical distribution (see Degtjareva et al., 2004, for details).

Degtjareva et al. (2004) have discussed and illustrated a high diversity of thickening pattern of anticlinal walls of the exotesta cells. The anticlinal walls may be thickened or not thickened in their interior, middle, and/or outer parts (Figs. 5F, G). These features are stable within each taxon investigated and should be quite useful for seed identification purposes, as well as for species-level taxonomy. However, they are highly homoplastic at the genus level.

Our data strongly suggest homoplasy in evolution of the cotyledon number in *Pinguicula*. This phenomenon can be easily obtained from all phylogenetic trees presented in this study (cotyledon numbers are indicated in parentheses after taxon names in Figs. 1, 3, 4, 7). Fig. 7 shows an unrooted tree inferred

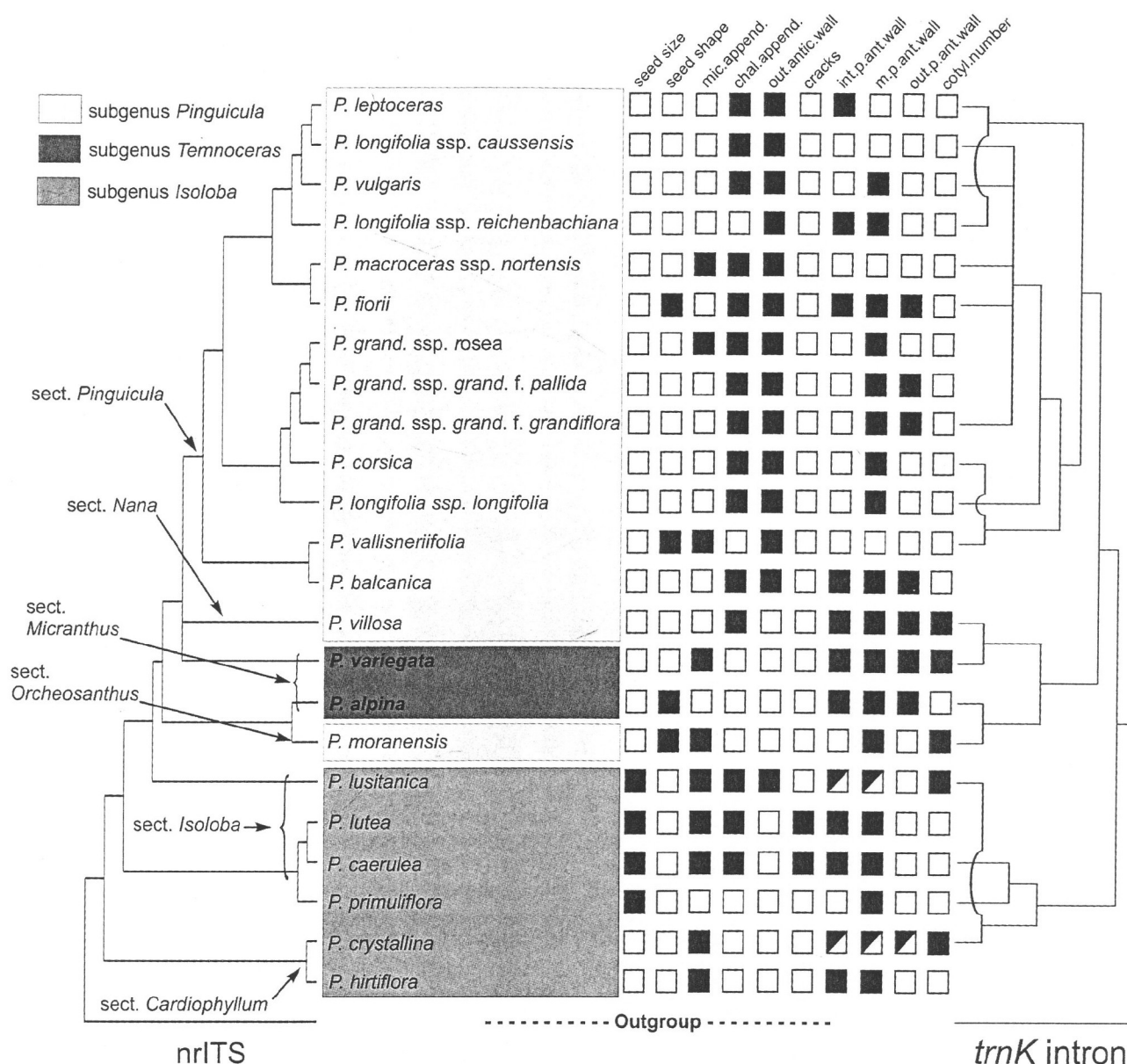


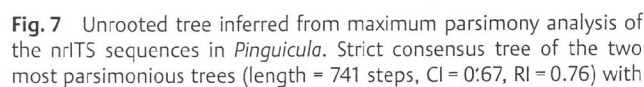
Fig. 6 Variation of the seed structure in the genus *Pinguicula* compared to the nrITS phylogeny (present study) and chloroplast phylogeny (Cieslak et al., 2005). ITS phylogeny (topology of the Bayesian tree in the Fig. 1 is on the left side while chloroplast phylogeny (also based on a Bayesian tree topology) is on the right side. The taxonomy of the genus *Pinguicula* according to Casper (1966) is also illustrated. The arrows show sections and the colour of the panels shows the subgenera. Character legend: seed size = seeds less than 0.6 mm long (black squares) or more than 0.6 mm (white squares); seed shape = spindly (black squares) or elliptic (white squares); mic. append. = micropylar appendage, less than 1/6 of seed length (black squares) or more than 1/6 of seed length (white squares); chal. append. = chalazal appendage, absent (black squares) or present (white squares); out. antic.

wall = outer parts of anticlinal walls of adjacent exotesta cells, more or less free and divided by a furrow (black squares) or almost completely united (white squares); cracks = cracks in outer periclinal cell walls of exotesta cells in mature seeds, present (black squares) or absent (white squares); int. p. ant. wall = interior part of anticlinal walls of exotesta cells, not thickened (black squares) or thickened (white squares), or polymorphism (black and white); m. p. ant. wall = middle part of anticlinal walls of exotesta cells, thickened (white squares) or not thickened (black squares), or polymorphism (black and white); out. p. ant. wall = outer part of anticlinal walls of exotesta cells, not thickened (black squares) or thickened (white squares), or polymorphism (black and white); cotyl. number = cotyledon number in embryo, two (black squares) or single (white squares).

from nrITS data in *Pinguicula*. It is clear that any kind of rooting of *Pinguicula* implies homoplasy in embryo evolution.

Homoplasy in embryo evolution is of special interest, because it seems that members of all related families, such as Gesneriaceae, Scrophulariaceae, Martyniaceae, Orobanchaceae, Glob-

ulariaceae, and Lamiaceae, possess embryos with two cotyledons. Data on cotyledon number cannot be aligned with our nrITS phylogeny. They are also neither congruent with the chloroplast phylogeny of Cieslak et al. (2005) nor with Casper's (1966) classification of the genus. We strongly suggest that homoplasy in embryo evolution of *Pinguicula* is natural and not



bootstrap support of nodes (1000 replicates). BS is omitted when it does not exceed 50%. The cotyledon number is given in parentheses after taxon names.

Whether cotyledons of *Pinguicula* are homologous to cotyledons of other dicots is still open to debate. Morphologically, cotyledons of *Pinguicula* are very simple structures (Figs. 5H,I), at least before seed germination, which makes their homologisation difficult. Although the vast majority of authors reject Treviranus' (1848) viewpoint that the so-called single cotyledon of *P. vulgaris* is in fact the first eophyll, it seems that no strong arguments against this theory have been found. Studies of *laterne* mutants may help to solve the problem (e.g., Burger, 1998). In the *laterne* phenotype of *Arabidopsis* (which is caused by a combination of two mutations), the cotyledons are deleted but the hypocotyl and root are unaffected (Trembl et al., 2005). Burger (1998) suggested that presence of *laterne* mutants supports earlier theories (e.g., Kudryashov, 1964) of the evolutionary origin of monocotyledons. This implies a complete loss of cotyledons and homology between the so-called cotyledon of monocotyledons and the first eophyll of dicotyledons. *Laterne* mutants of *Arabidopsis* are quite variable in number, structure, and arrangement of first eophylls. The number of first leaf primordia ranges from one for a single leaf, which sometimes develops a fused cup-shaped appearance, to numerous, which simultaneously develop leaf primordia (Trembl et al., 2005). The latter case may be compared to multiple cotyledon-like appendages described in embryos of some *Utricularia* species (e.g., Goebel, 1932; T aylor, 1994).

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