Two new species of the carnivorous genus *Pinguicula*, (*Lentibulariaceae*) from Mediterranean habitats

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Abstract: Two species of *Pinguicula* (*P. submediterranea* and *P. mundi*) are newly described from the south and east of the Iberian Peninsula. We analysed morphometric, cytogenetic, RAPD, ecological, and breeding system data to support the distinctness of these taxa. *Pinguicula submediterranea* and *P. mundi* are both hexaploid (2n = 48), a chromosome number previously not reported for the genus. Biometric, cytogenetical and molecular results distinguish *P. submediterranea* and *P. mundi* from other *Pinguicula* species, and from each other. *Pinguicula mundi* is predominantly outbreeding, whereas *P. submediterranea* both inbreeds and outbreeds. *Pinguicula submediterranea* and *P. mundi* appear to be isolated reproductively, although gene flow between the allopatric *P. submediterranea* and *P. mundi* is unlikely. Both *P. submediterranea* and *P. mundi* are threatened with extinction because suitable habitats are diminishing in size, or even disappearing, due to current aridity in the region.

Of the 250,000 or more species of flowering plants described to date, only about 500 are carnivorous. These belong to 19 genera in 9 families (Juniper & al. 1989). Because of their bizarre traits, carnivorous plants have long attracted the attention of researchers (e.g., Darwin 1874). The carnivorous trophic habit and its polyphyletic origins have been studied evolutionarily (Givnish 1989, Albert & al. 1992). However, little is known about the genetics, reproductive biology or ecology of most carnivorous species. Basic information such as chromosome number is lacking for most species, and the taxonomic affiliation of many species remains uncertain (Juniper & al. 1989).

The genus *Pinguicula* comprises small insectivorous, scapose and perenial herbs, with leaves typically in a basal rosette, zygomorphic flowers and capsular fruits. The leaves produce a sticky glandular secretion able to capture and digest small arthropods. The genus *Pinguicula* L. is one of the most widespread carnivorous groups, being present on all continents except Australia. There are about 35 *Pinguicula* species described to date (Casper 1966, Givnish 1989). Like most carnivorous plants, *Pinguicula* species are restricted to nutrient-poor habitats, such as bogs and swamps, which remain sunny and moist at least during the growing sea-

son (Givnish 1989). Despite the rarity of such sites in Mediterranean and similar ecosystems, many *Pinguicula* species are known from the Mediterranean Basin (Casper 1972) and semiarid habitats of Mexico and California (Givnish 1989, Studnicka 1991). In these regions, where plant growth is strongly limited by water availability, suitable habitats for *Pinguicula* species represent scattered, isolated wet areas surrounded by larger dry habitats. The current aridity of the Mediterranean Basin has made these small, isolated populations vulnerable to extinction. It is urgent to characterize these imperiled groups taxonomically as well as to identify the genetic and ecological factors that determine the viability and long-term conservation of populations of these rare species (Falk & Holsinger 1991, Schemske & al. 1994).

In this paper we describe two new species of the genus *Pinguicula* from a Mediterranean environment in the south and east of Spain: *P. submediterranea* and *P. mundi*. We have followed an interdisciplinary approach analysing: (1) the morphometry and cytogenetics of the two new species in comparison with congeners of the same geographical area; (2) random amplified polymorphic DNA (RAPD) analysis of the two new species in comparison with the putatively most closely related species; (3) the ecological conditions where the plants grow and (4) crossing experiments within populations, between populations of the same species, and between the two new species.

Material and methods

Cytogenetics and molecular analysis, as well as morphometric measurements and cross-pollination experiments were performed with plants cultivated from wild populations. Winter buds of *Pinguicula* species were obtained from several populations in the south, east and north of Spain in December 1992 (Table 1). Winter buds were stored at 4 °C until the beginning of March 1993, when they were planted in plastic containers, using a mixture of sand and cotton as a substrate. Plants were grown under natural light and constant watering regime, and at the beginning of the growing season were fed three flies (*Drosophila melanogaster*, wild race) per week. Plants grew normally and reached a mature size similar to that of wild plants. The species and populations studied are listed in Table 1.

Cytogenetic analysis. Mitotic analyses were made with root meristems of young plants, which were squashed in acetic orcein (Darlington & La Cour 1969).

RAPD Analysis. *Pinguicula submediterranea* and *P. mundi* plus *P. vallisneriifolia* and *P. grandiflora* were analysed using RAPDs. The first three species overlap in geographical distribution and share some ecological characteristics. Also, both *P. vallisneriifolia* and *P. grandiflora* have been repeatedly confused with the two new species. The intraspecific differences were analysed using two *P. submediterranea* populations (the most widely separated ones, 37731 from Granada and 37736 from Teruel), and two populations of *P. vallisneriifolia* (also the most distant ones, 37737 from Granada and 37738 from Jaén).

Approximately 1 g of leaf tissue was sampled in the field from four to six individual plants per population for genomic DNA extraction as described by Dellaporta & al. (1983), but including a final purification step with the Magic DNA clean-up system (Promega). A set of six 10-bp oligonucleotides purchased from Isogen Bioscience Bv. (Amsterdam, Netherlands) were used as simple primers (Table 2) for DNA amplification. Amplification reactions were performed in a volume of 25 µl of mix containing 10mM Tris HCl at pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 200 µM each of dATP, dCTP, dGTP, and dTTP, 50 ng of genomic DNA, 40 ng of primer and 1U of Taq DNA polymerase (Promega). The reaction mix was overlaid with mineral oil. After an initial denaturing of DNA

Table 1. The *Pinguicula* species and populations studied. In the case of plants growing on rocky substrates, we collected where possible winter buds fallen from the rocky wall due to effects of snow and ice. From ground plants, we collected specimens uprooted by large herbivores, as well as axillary buds (voucher specimens in the Herbarium of the Faculty of Sciences, University of Granada, GDAC)

Species	Locality	Voucher
Pinguicula grandiflora Lam. subsp. *grandiflora*	Asturias: Picos de Europa 1100 m s.m.	GDAC-37723
	Asturias: Cangas de Onís, 500 m s.m.	GDAC-37724
	Huesca: Plan 1200 m s.m.	GDAC-37725
	Navarra: Sierra de Aralar 1150 m s.m.	GDAC-37726
Pinguicula longifolia Ramond ex DC. subsp. *longifolia*	Huesca : Añisclo, 1050 m s.m.	GDAC-37727
	Huesca: Plan, 1150 m s.m.	GDAC-37728
Pinguicula mundi, spec. nova	Albacete: Riópar, 1200 m s.m.	GDAC-37729
Pinguicula nevadensis (Lindb.) Casper	Granada : Sierra Nevada, 2500 m s.m.	GDAC-37730
Pinguicula submediterranea, spec. nova	Granada : Sierra Tejeda, 1700 m s.m.	GDAC-37731
	Jaén : Sierra de Cazorla, 1300 m s.m.	GDAC-37732
	Jaén : Sierra de Cazorla, 1400 m s.m.	GDAC-37733
	Jaén: Sierra de Segura, 1400 m s.m.	GDAC-37734
	Tarragona: Pto. Tortosa, 800 m s.m.	GDAC-37735
	Teruel : Pto. Beceite, 900 m s.m.	GDAC-37736
Pinguicula vallisneriifolia Webb	Granada: Sierra de Almijara, 900 m s.m.	GDAC-37737
	Jaén: Sierra de Segura, 1100, m s.m.	GDAC-37738
	Jaén: Sierra de Cazorla, 1400 m s.m.	GDAC-37739
	Jaén: Sierra de Segura, 1200 m s.m.	GDAC-37740
	Jaén: Sierra de Segura, 1300 m s.m.	GDAC-37741
	Jaén : Sierra de Segura, 900 m s.m.	GDAC-37742

at 94 °C for 10 min, amplification was performed in a thermocycler (Pharmacia) for 40 cycles of 1 min at 94 °C, 1 min at 37 °C and 1.5 min at 72 °C. Amplified DNA was resolved on 1.5% agarose gel in TAE buffer, stained with ethidium bromide and photographed.

A total of 90 different bands were scored as present (1) or absent (0) for all the populations studied. Pairwise comparisons of populations based on presence or absence of bands were used to determine values of genetic distances between them. The genetic distances were calculated as the proportion of total number of bands scored that were different between each pair of populations (Wilke & al. 1993). The genetic matrix generated was then used as input data for cluster analysis by UPGMA, using the microcomputer package PHYLIP (Felsenstein 1993).

Morphology and biometrics. Morphometric analyses were carried out with five to ten flowering plants of the species and populations mentioned above. Seeds were photographed using scanning electron microscopy (SEM), after first coating them with a thin layer of gold-paladium (Thornill & al. 1965).

Table 2. Name of primers used in this study, sequences, and number of bands produced from each population after amplification. Populations: 1 *Pinguicula vallisneriifolia* 37738; 2 *P. vallisneriifolia* 37737; 3 *P. submediterranea* 37731; 4 *P. submediterranea* 37736; 5 *P. mundi* 37729; 6 *P. grandiflora* 37726

Primer	Sequence	1	2	3	4	5	6	Total
3764	AGGCCCGATG	3	4	3	3	8	8	14
3768	AAGGCTCACC	6	6	7	7	10	5	21
3770	GGCTGGTTCC	5	5	5	4	4	9	17
3771	TGGGTCCCTC	1	1	1	1	1	1	1
3772	AGATCCCGCC	4	4	6	5	9	7	18
3773	GAGTGCCGGT	6	2	5	6	5	4	19

In the field, we quantified several phenotypic traits of the two new species related to vegetative growth, and sexual and asexual reproduction in flowering and fruiting phenophases. We also estimated the number of reproductive plants per population. Field samples used the only known population of *P. mundi* (37729 from Albacete), as well as the Granada population (37771), and two Jaén populations (37733 and 37734, respectively) of *P. submediterranea*.

Breeding system. Plants with flowers in the bud stage were covered with nylon-mesh bags (0.2 mm mesh), allowing air flow and transpiration, but excluding pollinators. Netted plants were checked daily to ensure full pollinator exclusion. Hand-selfing and hand-crossing was carried out with *P. mundi* and *P. submediterranea* to test: (1) spontaneous autogamy in the absence of insect vectors; (2) self-fertilization by hand with pollen from the same flower; (3) cross-fertilization by hand with a pollen donor belonging to the same population; (4) cross-fertilization by hand with a pollen donor belonging to a different population (only for *P. submediterranea*). In *P. submediterranea*, plants were crossed from three populations (37731 from Granada, 37733 from Jaén, and 37735 from Tarragona).

Cultivated plants flowered nearly synchronously, allowing a significant number of flowers to be hand-crossed. A total of 20 reproductive specimens per population were crossed, using five reproductive plants per experimental cross and per population as pollen receptors and donors (see Table 7). Another five reproductive plants per population were left untouched as a control group for spontaneous autogamy.

To test the possibility of gene flow between the two new species, we crossed *P. mundi* (five reproductive plants) × *P. submediterranea* (ten reproductive plants, 37731 from Granada and 37735 from Tarragona populations) as pollen donor.

We hand-pollinated using a fine brush, touching whole anthers of the pollen donor (and thus collecting most of the pollen available) and then touching the stigma of the corresponding receptor plant. Seeds were harvested on the day the capsules dehisced, and the number of seeds and aborted embryos or ovules were counted in the laboratory with a binocular microscope to determine the seed:ovule ratio. Due to the low weight of individual seeds, only mean seed weight per capsule was calculated.

Results

Morphology. The key morphometric traits for distinguishing the two new species, *P. submediterranea* and *P. mundi*, and their closest relatives, *P. grandiflora* and *P.*

Table 3. Principal features of the two new *Pinguicula* species and closely related species

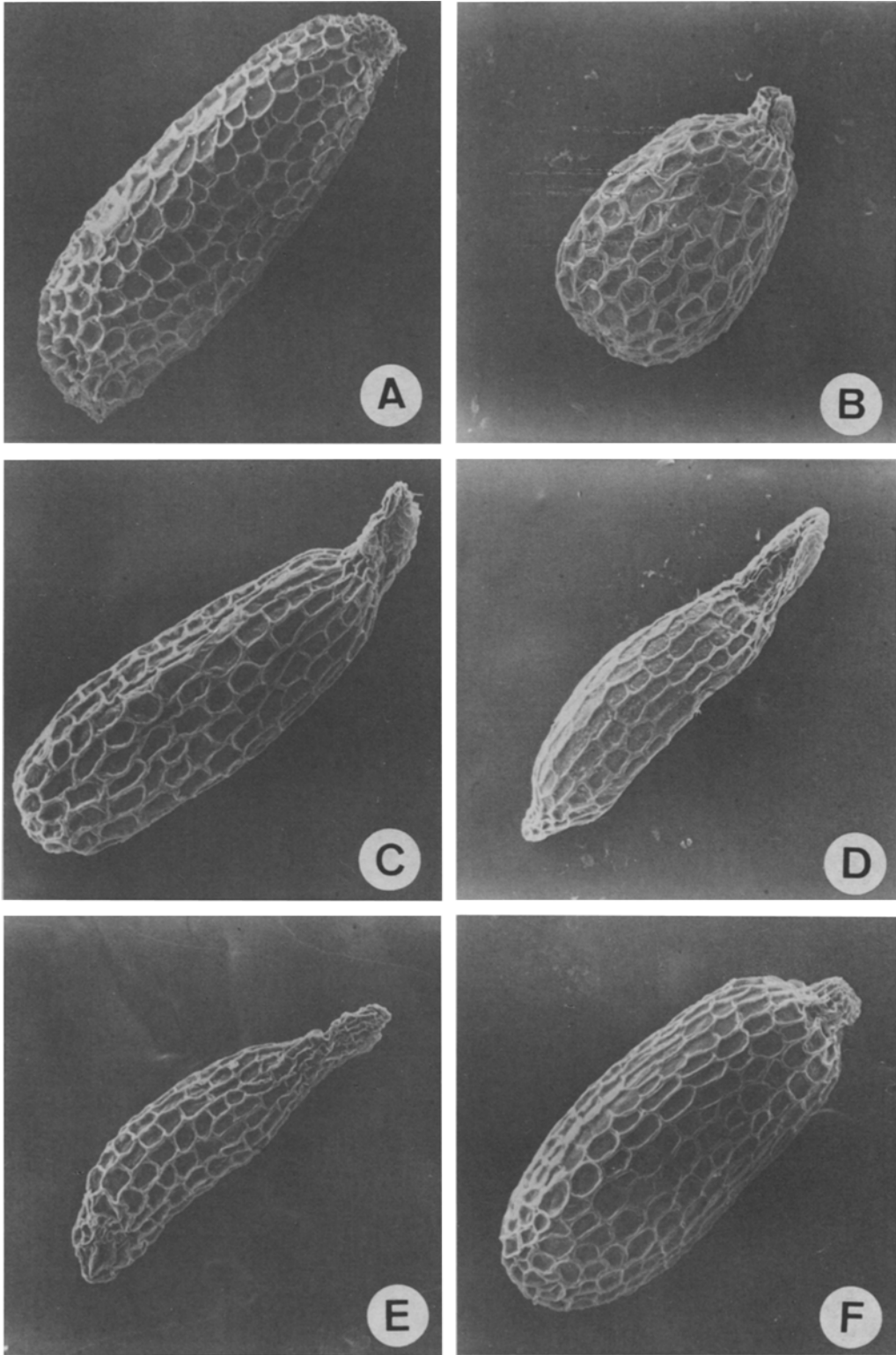| Traits	*P. grandiflora*	*P. submediterranea*	*P. mundi*	*P. vallisneriifolia*
Spring leaves (anthesis)	horizontal, close to the soil	horizontal, close to the soil	suberect	suberect to erect
Summer leaves (fruiting)	similar to spring leaves	similar to spring leaves	suberect, longer than spring leaves, elliptic to obovate	suberect to erect, much longer than spring leaves, ligulate, undulate in the margins
Upper calyx lobes	4–5 mm, oblong, elliptic or subtriangular	(2-) 2.5–3.5 mm, ovate-lanceolate to lanceolate	2.5–3.5 mm, triangular-obtuse or slightly elliptic	3–5 mm, ovate-oblong to ovate-suborbicular
Corolla length Corolla colour	17–20 mm entirely violet	(12-) 13–17 (-18) mm violet, lobes of the lower lip coloured only in the distal half	(12-) 14–18 (-19) mm violet, sometimes with veins weakly marked, lobes of the lower lip coloured only in the distal half	(14-) 15–22 mm with violet veins, pale violet to pinkish in the distal part of the lobes, rarely almost white
Corolla palate	white, with two dark spots in the upper part, near to the stigmate	dark violet, except in the middle lobe of the lower lip	dark violet, except in the middle lobe of the lower lip	white
Corolla spur	(11-) 12–16 mm	(6-) 7–11 (-13) mm	(8) 9–12 (-13) mm	(9-) 11–18 mm
Seed 2n	narrowly ellipsoidal 32	ovoidal 48	narrowly ellipsoidal 48	clavate 32

Fig. 1. Seeds photographed with a scanning electron microscope. *A Pinguicula grandiflora; B P. submediterranea; C P. mundi; D P. vallisneriifolia; E P. longifolia; F P. nevadensis.* Bar: 100 μm

Table 4. Phenotypic and reproductive traits of *Pinguicula mundi* and *P. submediterranea* natural populations. Spring and summer leaf measurements refer to the length of the longest leaf per reproductive plant. Spring and summer angle were calculated as the angle of leaf growth with respect to vertical at the centre of the rosette (e.g., $0°$ = vertical, erect leaf; $90°$ = horizontal, flattened leaves forming a typical rosette). Sample sizes (n) refer to number of reproductive plants analysed per population. Values are given as means ± SE. *** $p < 0.0001$ (all one-way ANOVAs were performed with log-transformed data, except fruit:flower ratio variable, which was arcsin-transformed)

| Species and populations	Spring leaves (mm)	Summer leaves (mm)	Spring leaf angle (°)	Summer leaf angle (°)	No. of flowers per plant	Fruit:flower ratio (%)	% of plants with stolons
P. mundi							
37729 (n = 40)	47.4 ± 1.3	78.4 ± 3.3	72.7 ± 2.5	63.5 ± 4.1	3.6 ± 0.2	76.8 ± 6.3	40
P. submediterranea							
37731 (n = 40)	52.3 ± 2.0	57.5 ± 2.1	83.7 ± 1.8	85.3 ± 2.6	2.1 ± 0.1	63.5 ± 6.41	6
37733 (n = 20)	48.4 ± 1.9	52.4 ± 2.0	88.7 ± 0.8	87.7 ± 0.8	3.1 ± 0.2	68.5 ± 4.97	4
37734 (n = 25)	50.2 ± 10.3	52.2 + 1.6	84.6 ± 2.0	86.6 ± 2.3	2.4 ± 0.2	58.6 ± 4.32	2
F	1.637, ns	24.85***	10.26***	20.26***	10.68***	14.42***	
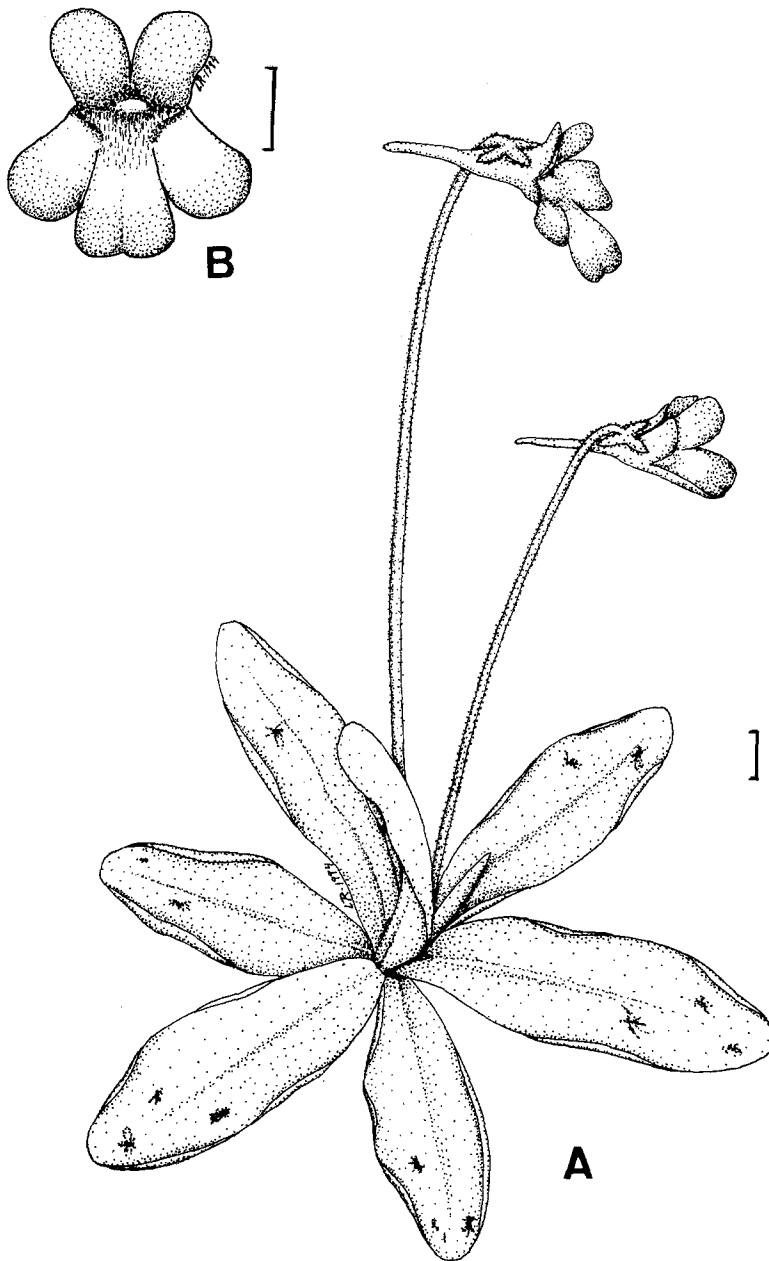
Fig. 2. *Pinguicula submediterranea*. *A* Habit; *B* flower. Bars:1 cm

vallisneriifolia (respectively), appear in Table 3. *P. submediterranea* differs from *P. grandiflora* in that the former has small flowers (see measurements for calyx-lobes, corolla length and corolla spur) with different colour patterns (Table 3). In addition, *P. grandiflora* seeds (Fig. 1 A) are narrowly ellipsoidal, and the seed coat is ornamented with isodiametric and/or elongated reticulum units, whereas the seeds of *P. submediterranea* (Fig. 1 B) are consistently ovoid with elongated reticulum units, more irregular in form and in spatial distribution.
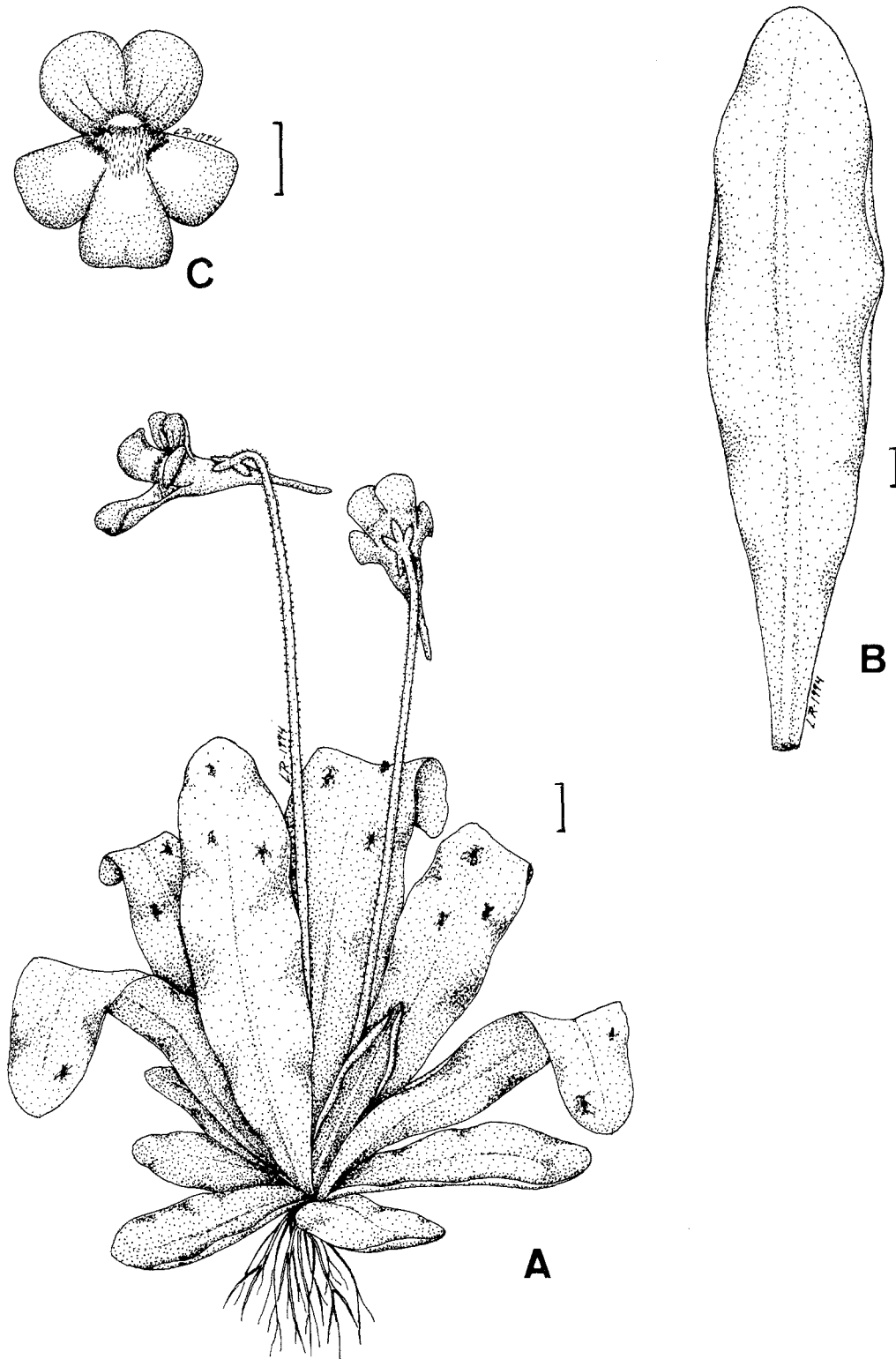
Fig. 3. *Pinguicula mundi*. *A* Habit; *B* summer leaf; *C* flower. Bars: 1 cm

P. mundi differs from *P. vallisneriifolia* in that the summer leaves of the former are elliptical to ovate (versus ligulate), and the flowers are smaller with a dark violet corolla palate, except in the middle lobe of the lower lip (versus with violet veins). *P. mundi* seeds (Fig. 1 C) are narrowly ellipsoidal whereas the seeds of *P. vallisnerifolia* (Fig. 1 D) are clavate, like the seeds of *P. longifolia* (Fig. 1 E). On the other hand, *P. nevadensis* (Fig. 1 F) seeds are ellipsoidal, unlike those of the other species.

P. mundi and *P. submediterranea* have similar spring leaves, both in form and size, although those of *P. mundi* are slightly suberect (Table 4). Furthermore, *P. mundi* has larger, suberect summer leaves. By contrast, *P. submediterranea* consistently has a rosette of summer leaves close to the substrate, spring and summer leaves being very similar in size, form and spatial distribution (Table 4, Fig. 2). The leaves from both species are flat, although in *P. submediterranea* the leaf margins curve slightly inwards. The suberect summer leaves of *P. mundi*, however, have wavy margins curving outwards (see Fig. 3).

The flower of *P. mundi* is larger than that of *P. submediterranea* (Table 3). Both species have a straight spur, slightly longer in *P. mundi*. The corolla shape and colour are very similar in both species: violet-purple at the tip of the petals, contrasting with the white in the middle of the petals and, again, strong violet-purple colouration on the palate at the base of the petals. Some *P. mundi* flowers (nearly 10%) have floral guides. This floral trait is absent in *P. submediterranea,* but is common in *P. vallisneriifolia* (Table 3).

With respect to asexual reproduction, 40 % of reproductive wild plants of *P. mundi* developed stolons (range 1–3 per plant), invariably less than 1.5 cm in length (Table 4). This kind of vegetative reproduction is rare in *P. submediterranea* (only 4% of reproductive plants developed stolons).

The phenology of *P. submediterranea* has been studied in the 37731 population from Granada (1700 m s.m.). Plants overwinter as buds, which start to grow in May, forming a rosette of basal leaves. Flowering occurs at the end of June, and fruiting from July to the beginning of August. Leaves senesce from August to September, coinciding with the formation of the central and axillary winter buds. On the other hand, *P. mundi* plants (37729 from Albacete) also overwinter as buds and start growing in April, forming a rosette of basal leaves. Flowering occurs at the end of May or the beginning of June, and fruiting from June to July, simultaneously with suberect leaves and stolons development. Leaves senesce from August to September, when central and axillary winter buds begin to form.

Cytogenetic analysis. *P. submediterranea* and *P. mundi* are both hexaploid (2n = 48), a previously unreported chromosome number for the genus. The other species analysed have different ploidy levels: *P. grandiflora, P. vallisneriifolia,* and *P. longifolia* are tetraploids (2n = 32), whereas *P. nevadensis* is diploid (2n = 16), also a previously unreported chromosome number (Table 5).

Molecular analysis. Random amplified polymorphic DNA (RAPD) markers were used to evaluate the differences between species and between populations of some *Pinguicula* species. Six 10-mer oligonucleotides were selected for amplifying the DNA (Table 2). With the exception of one of the primers (3771) which gave rise to a unique band in all the species analysed, the remaining primers gave complex banding patterns with a considerable variation at both the interspecific

Table 5. Chromosome numbers of the *Pinguicula* populations studied

Species and voucher specimens	2n	Previous counts
P. grandiflora		2n = 32: Doulat (1947), Wood & Godfrey (1957), Contandriopoulos (1962), Casper (1963)
GDAC 37723	32	
GDAC 37724	32	
GDAC 37725	32	
GDAC 37726	32	
P. submediterranea		
GDAC 37731	48	
GDAC 37733	48	
GDAC 37735	48	
GDAC 37736	48	
P. mundi		
GDAC 37729	48	
P. vallisneriifolia		2n = 32: Löve & Kjellqvist (1974)
GDAC 37737	32	
GDAC 37738	32	
GDAC 37739	32	
GDAC 37740	32	
GDAC 37741	32	
GDAC 37742	32	
P. longifolia		2n = 32: Doulat (1947), Casper (1962, 1963)
GDAC 37727	32	
GDAC 37728	32	
P. nevadense		
GDAC 37730	16	

and the intraspecific level (Fig. 4). The number of amplification products varied from 1 to 21 with a size range from 50 to 2000 bp. A total of 90 RAPD bands were scored among all the populations analysed (Table 2).

The banding patterns of each primer were used to generate a matrix of presence-absence for each band in each population, and to calculate the genetic distance between the following populations: 37738 from Jaén and 37737 from Granada (*P. vallisneriifolia*), 37731 from Granada and 37736 from Teruel (*P. submediterranea*), 37729 from Albacete (*P. mundi*), and 37726 from Navarra (*P. grandiflora*). Genetic distance were arranged in matrix form (Table 6) and subjected to UPGMA analysis to generate the phenogram in Fig. 5.

Breeding systems. *Pinguicula submediterranea* and *P. mundi* exhibited no spontaneous autogamy. However, all experimental hand-crosses resulted in a high proportion of flowers setting fruit (Table 7). For *P. submediterranea,* two-way
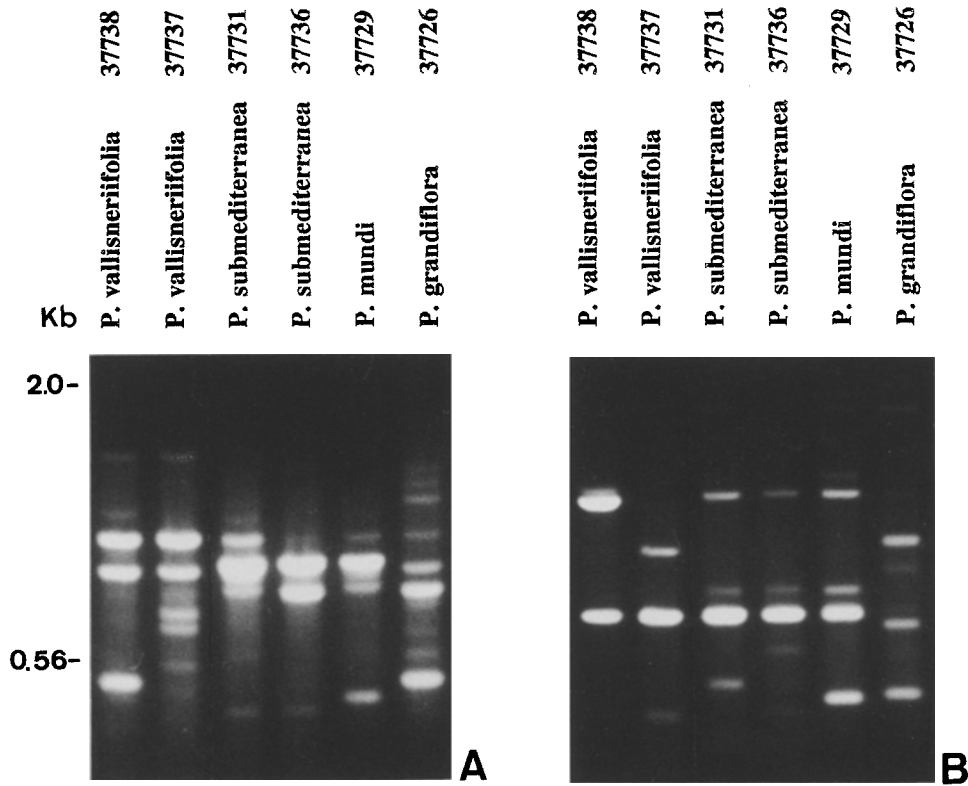
Fig. 4. Amplification products of primers 3770 (*A*) and 3772 (*B*) in *Pinguicula*

Table 6. Matrix of genetic distances between some populations of *Pinguicula*

	P. vallisnerii-folia 37737	P. submedi-terranea 37731	P. submedi-terranea 37736	P. mundi 37729	P. grandi-flora 37726
P. vallisneriifolia 37738	0.482	0.702	0.763	0.770	0.854
P. vallisneriifolia 37737	–	0.736	0.763	0.744	0.804
P. submediterranea 37731		–	0.542	0.659	0.842
P. submediterranea 37736			–	0.760	0.884
P. mundi 37729				–	0.771

Table 7. Results of the experimental hand-crosses. Sample sizes (n) refer to number of flowers crossed per population. Seed:ovules ratios, seed number and seed weight values are given as means ± SE. Ovule number for *Pinguicula submediterranea* is: Granada population (37731) = 135.68 ± 7.01 (n = 31); Jaén population (37733) = 82.57 ± 6.77 (n = 7); Tarragona population (37735) = 98.45 ± 8.33 (n = 20). Ovule number for *P. mundi* is : 165 ± 9.9 (n = 16). See text for statistical analysis

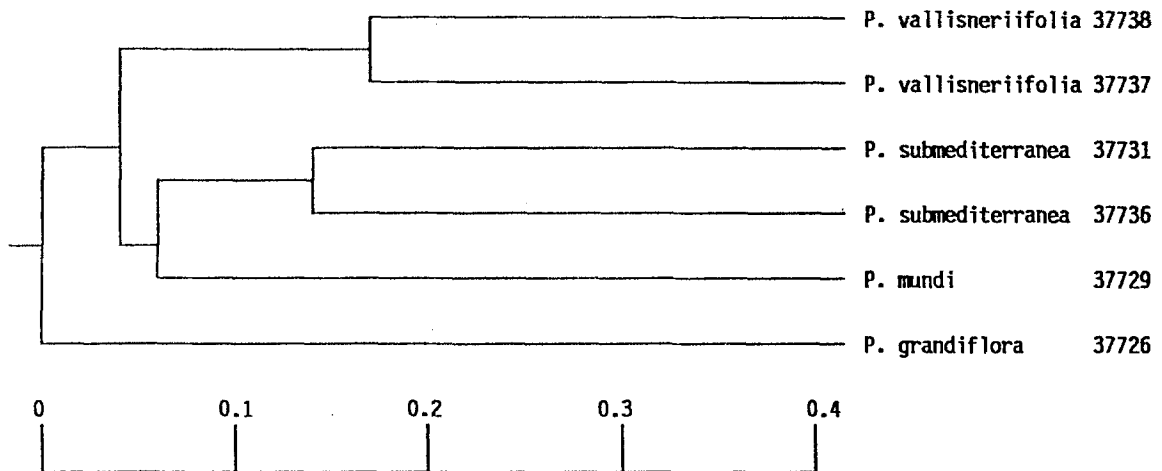| Species	Population (pollen receptor)	Pollen donor	Fruit:flower ratio (%)	Seed : ovules ratio (%)	Seed number per capsule	Mean seed weight (mg)
P. submediterranea	Tarragona 37735	Autogamy (10)	0	—	0	—
		Selfing (8)	87.7	48.2 ± 9.9	54.7 ± 12.2	0.015 ± 0.001
		Xenogamy (5)	80	40.8 ± 7.4	34.3 ± 16.3	0.021 ± 0.004
		Jaén 37733 (5)	80	58.1 ± 15.2	71.0 ± 29.1	0.021 ± 0.002
		Granada 37731 (7)	85.7	73.7 ± 15.5	73.8 ± 12.5	0.017 ± 0.002
	Granada 37731	Autogamy (10)	0	—	0	—
		Selfing (7)	85.7	73.8 ± 6.9	98.4 ± 15.4	0.022 ± 0.001
		Xenogamy (14)	92.8	64.3 ± 5.6	85.3 ± 11.5	0.022 ± 0.001
		Jaén 37733 (7)	100	86.3 ± 1.0	130.4 ± 12.4	0.023 ± 0.001
		Tarragona 37735 (4)	100	58.1 ± 20.7	103.5 ± 48.2	0.026 ± 0.006
	Jaén 37733	Autogamy (6)	0	—	0	—
		Selfing (2)	100	76.5 ± 2.5	66 ± 9	0.018 ± 0.001
		Xenogamy (2)	100	76.5 ± 2.5	66 ± 9	0.020 ± 0.001
		Tarragona 37735 (2)	100	74.5 ± 6.0	64 ± 12	0.021 ± 0.001
		Granada 37731 (4)	100	59.3 ± 3.2	48 ± 8.4	0.020 ± 0.001
P. mundi	Río Mundo 37729	Autogamy (12)	0	—	0	—
		Selfing (13)	46.1	82.1 ± 4.6	122 ± 10.3	0.022 ± 0.001
		Xenogamy (10)	70	81.7 ± 5.9	170 ± 20.7	0.030 ± 0.001
		P. submediterranea Tarragona 37735 (6)	0	—	0	—
		P. submediterranea Granada 37731 (9)	33.3	44.9 ± 23.8	72 ± 38.1	0.019 ± 0.001

Fig. 5. Dendrogram generated by cluster (UPGMA) analysis of genetic distance values given in Table 6 showing relationships between different *Pinguicula* species

ANOVAs were performed using experimental cross (four levels, see Table 7) and population (two levels, Tarragona 37735 and Granada 37731; the Jaén population, 37733, was excluded because the sample size was too small for statistical analysis of hand-crosses, Table 7) as factors, to test the importance of population versus experimental cross in relation to several estimators of plant reproductive success. Population (Granada 37731 versus Tarragona 37735) was the only factor that significantly explained differences in seed number (F = 11.37, df = 1, 42, p = 0.0016, log-transformed data) and seed weight (F = 4.21, df = 1, 42, p = 0.0465, log-transformed data), experimental cross not being significant. These results indicate that seed number and weight in *P. submediterranea* were similar in selfing and outcrossing treatments, both within and between populations. Furthermore, both factors (population and experimental cross) affect the seed:ovule ratio (F = 7.75, df = 1, 42, p = 0.008, and F = 2.76, df = 4, 42, p = 0.04, respectively, arcsin-transformed data). The interaction terms in all of the two-way ANOVAs described above were not significant.

The three geographically distinct populations of *P. submediterranea* (Granada 37731, Jaén 37733, and Tarragona 37735) differ significantly in the number of ovules per flower (one-way ANOVA, F = 10.19, df = 2, 56, p = 0.002, log-transformed data), with the highest population, Granada 37731 (1700 m s.m.), having more ovules per flower than the others (Table 7).

The percentage of *P. mundi* flowers setting fruits is less when hand-selfed than when hand-crossed (Table 7). Furthermore, seed number and, above all, seed weight of *P. mundi* are greater in outcrossing than in selfing treatments (F = 3.08, p = 0.1, df = 1,11, and F = 37,96, p = 0.0001, df = 1, 11, respectively, log-transformed data).

Hand-crosses performed between *P. submediterranea* and *P. mundi* indicated a reproductive barrier between the two new species. The cross between *P. mundi* and *P. submediterranea* Tarragona population (37735, the most distant one), produced no viable seed, whereas with the Granada population (37731) of *P. submedi-*

terranea as a pollen donor, only 33% of crosses produced capsules (Table 7). The number and weight of seeds in crosses was lower than the corresponding number obtained with outcrossing and selfing in *P. mundi* (F = 4,84, p = 0.02, df = 2, 15, and F = 28,62, p = 0.0001, df = 2, 15, respectively, log-transformed data; see Table 7).

Discussion

Using morphometric, cytogenetic, molecular, and ecological data, *P. submediterranea* and *P. mundi* can be distinguished from the closely related *Pinguicula* species (*P. grandiflora* and *P. vallisneriifolia*), and from each other, as follows:

Morphology. *P. mundi* differs from *P. vallisneriifolia* in several key traits, such as the size and shape of the summer leaves, the size, colour and shape of the flowers, and seed characteristics (Fig. 1, Table 3). *P. submediterranea* clearly differs from *P. grandiflora* in flower size and colour, as well as in seed characteristics (Fig. 1, Table 3).

P. *mundi* is characterized by some heterophylly, with the summer leaves being larger than the spring ones, and clearly suberect. By contrast, *P. submediterranea* invariably shows a characteristic flat rosette of leaves. *P. mundi* also has greater vegetative and reproductive vigour than *P. submediterranea*, achieving a larger maximum size. *P. mundi* also develops stolons more frequently than *P. submediterranea*. Both species clearly differ with respect to seed characteristics (Fig. 1). On the other hand, flowers of *P. mundi* and *P. submediterranea* have similar pattern of contrasting colours, probably to attract the insects needed as pollen vectors.

Cytology. The hexaploid chromosome number (2n = 48) of *P. submediterranea* and *P. mundi* was previously unreported for any *Pinguicula* species. *P. grandiflora, P. vallisneriifolia, P. longifolia,* and *P. alpina* are tetraploids (2n = 32), whereas *P. nevadensis* is diploid (Table 5). *P. vulgaris* is the most complex species with respect to chromosome number (2n = 64; see for example Löve & Löve 1956, 1982; Wood & Godfrey 1957; Casper 1963; Laane 1969; Löve & Kjellqvist 1974; Murin 1976). However, Casper (1962) noted the tetraploid level (2n = 32). *P. alpina* is also tetraploid (2n = 32, see Casper 1962), while *P. lusitanica* is diploid, but with a different basic chromosome number (2n = 12, see Casper 1963). Thus, changes in ploidy level may be the basic mechanism for evolutionary diversification in the genus *Pinguicula*. In this respect, the two hexaploid novelties (*P. submediterranea* and *P. mundi*) probably are derived within their group. In fact, some information is available on interspecific hybrids in *Pinguicula* (Casper 1972). *P. mundi* very probably has an allopolyploid origin, from the crossing of a tetraploid species (2n = 32, probably *P. vallisneriifolia*) with a diploid one (2n = 16), and the subsequent duplication of the chromosomal number. The origin of *P. submediterranea* would be similar to that described for *P. mundi*, implying a tetraploid – probably *P. grandiflora*, the one genetically closest to *P. submediterranea* – crossing with an unknown diploid. Another possibility would be the result of the crossing of an octoploid species (2n = 64) and a tetraploid one (2n = 32); the two species involved may have been *P. vulgaris* and *P. grandiflora*, respectively. This latter possibility would be highly probable if *P. vulgaris* were an autopolyploid originating from *P. grandiflora*.

RAPDs. Recent works have shown RAPD analysis to be a useful tool for resolving classification problems in plants (BRAUNER & al. 1992,CRAWFORD & al. 1994, VAN BUREN & al. 1994). In the present study, intra-specific differentiation detected between the more distant populations of *P. vallisneriifolia* (0.482) and *P. submediterranea* (0.542) is lower compared with that found between species (values of genetic distances from 0.659 to 0.884, Table 6). Therefore, the RAPD data support the distinctness of these taxa based on morphological characteristics and breeding assays. *P. grandiflora* appeared to be the most differentiated among the species considered, while *P. vallisneriifolia* is the most related to the two new species (*P. submediterranea* and *P. mundi*). The two new species, although closely related, are also shown as distinct genetically from each other (Fig. 5).

Breeding systems. Both *P. submediterranea* and *P. mundi* are self-compatible but not autogamous, due to herkogamy. Both species depend on insect pollinators for sexual reproduction. With respect to this, fruit:flower ratios obtained by hand-crosses (Table 7) were higher than natural fruit:flower ratios (Table 4), indicating pollinator limitation in natural populations of *P. submediterranea*. By contrast, fruit:flower experimental (xenogamy treatment) and natural ratios in *P. mundi* were very similar. In fact, no pollinators were seen in any population of *P. submediterranea*, whereas *Bombyllidae* and small bees were occasionally seen visiting flowers of *P. mundi*.

Fruit:flower ratios and seed:ovules ratios were similar in outcross and selfed hand-crosses in *P. submediterranea* (Table 7). There is thus unlikely to be any inbreeding depression in this species in which natural seed set is presumably a mixture of self- and cross-fertilization. These results agree with theoretical models proposing that small populations with a long history of inbreeding would show little inbreeding depression (CHARLESWORTH & CHARLESWORTH 1987, KARRON 1991). By contrast, *P. mundi* is predominantly an outbreeder, though also able to inbreed.

Despite geographical isolation, all populations of *P. submediterranea* share a common genetic pool (i.e., according to the biological species concept; MAYR 1992). Crosses performed between plants belonging to different populations of *P. submediterranea* showed similar or even greater success (considering the number of seeds per capsule; Table 7) than the corresponding crosses performed between plants belonging to the same population. Thus, there is no indication of outbreeding depression in *P. submediterranea*. These results may indicate a loss of genetic variation within populations as a consequence of repeated crosses between relatives.

P. submediterranea and *P. mundi* appear to be isolated by strong reproductive barriers, reflected by the fact that hand-crosses performed produced either no mature capsules (*P. submediterranea*, Tarragona population as pollen donor) or only a small proportion of mature capsules with few, very small seeds (*P. submediterranea*, Granada population as pollen donor). Furthermore, there is no opportunity for gene flow between *P. submediterranea* and *P. mundi* because of the current allopatric distribution of the two species.

The Mediterranean ecological theatre and the viability of rare *Pinguicula* species. *P. mundi* and *P. submediterranea*, like most *Pinguicula* species, are restricted to habitats which remain wet during the growing season (spring and summer). *P. mundi* occupies wet rocky habitats (walls and slopes), whereas *P. sub-*

mediterranea populations occur both on wet rocky walls and ground microsites. Because of the dry Mediterranean summer, these wet sites attract many herbaceous and woody plant species; and are habitable for only a short duration because of plant successional displacement. *Pinguicula* plants can escape overgrowth competition by colonizing wet rocky substrates (open gaps free of plant competition), unsuitable for plants with more developed root systems. The carnivore habit can be considered as a pre-adaptation for a successful colonization of rocky substrates which are only suitable for plants with rudimentary roots, such as *Pinguicula*.

The pronounced habitat specificity of *P. submediterranea* and *P. mundi* has evolutionary consequences, since suitable wet habitats are equivalent to islands of moisture surrounded by a sea of dryness. Furthermore, local population size is seriously constrained by the limited space of suitable habitats. These species represent two different kinds of rarity (sensu Ravinowitz 1981). *P. mundi* is restricted to the head-water of the Mundo River (one population = one species), where the species is locally abundant (more than 10,000 reproductive plants). This population is 50 km from the nearest *P. submediterranea* population, whereas populations of *P. vallisneriifolia* are found 18 km west and 10 km south, respectively. *P. submediterranea* by contrast has a wide, but highly scattered geographic distribution (the most distant populations being 700 km from each other), consistently showing small population sizes (less than 500 plants and normally between 20 and 200 plants per population), occupying only a few square meters each. These populations appear to be the last survivors of a species broadly distributed throughout the Mediterranean Basin during the wetter Quaternary periods. Small remnant populations of *P. submediterranea* are likely to suffer genetic bottlenecks and loss of diversity both because of repeated mating between relatives over a long period, and because of the potential for selfing (Barret & Kohn 1991). Populations of *P. submediterranea* also have a high extinction probability because their small size and very limited spatial distribution promotes demographic and environmental stochasticity (Gilpin & Soule 1986, Menges 1991). Furthermore, despite the high fruit and seed set per reproductive plant and the possibility of asexual reproduction, *P. mundi* and *P. submediterranea* are threatened with extinction because the size of suitable wet sites is progressively diminishing, (or even disappearing) due to the current climatic aridity. In conclusion, the survival of *P. mundi*, and especially, *P. submediterranea* populations in a long-term perspective may depend on a future glaciation.

Pinguicula submediterranea G. Blanca, M. Jamilena, M. Ruiz-Rejón, R. Zamora, spec. nova (Fig. 2)

= *P. grandiflora* Lam. var. *dertosensis* Cañigueral, Collect. Bot. (Barcelona) **5**: 413 (1957), ex loco!, excl. descr. pro parte (Type: "Puertos de Beceite, in confinibus Aragoniae et catal. pr. Font del Teix, in initio Barranc del Parrissal, aprilis 1935, legit Bartomeus (BC-87830)".

Planta quam *P. grandiflora* minor, calycis lobis superis (2–) 2,5–3,5 mm longis, lanceolatis vel ovato-lanceolatis, corolla (12–) 13–17 (–18)mm – huius autem calcar (6–) 7–11 (–13)mm, lobuli inferiores apice tantum colorati atque faux intense

violacea praeterquam quoad inferioris labii lobulum centralem–, seminibus ovoideis et chromosomatum numero 2n = 48.

Holotypus: Sierra de Cazorla (Jaén provincia, Hispania), 1300 m s.m. alt. in clivis terreis humidissimis atque muscis coopertis, ubi legit R. Zamora, 10-VI-1993 (GDAC 37732).

English description. Small insectivorous, scapose and perennial herbs with slender roots, overwintering as a bud. Leaves 6–9 (–12), in a basal rosette, horizontal, close to the soil, entire, shortly petiolate, soft and fleshy, clothed with viscid glands above, with the margin more or less involute; spring leaves (anthesis) (2–) 3–6 × 1.5–2.5 cm, obovate to obovate-oblong; summer leaves (fruiting) similar to spring leaves. Flowers solitary, zygomorphic; pedicels 1–5. 5–12 cm long, naked, covered with viscid glands, the stalks not more than twice as long as the gland. Calyx persistent, 2-lipped, with viscid glands; upper lip 3-lobed, with lobes (2–) 2.5–3.5 mm long, ovate-lanceolate to lanceolate, obtuse or subacute; lower lip 2- lobed, divided for up to 1/2 its length or sometimes nearly to the base. Corolla (12–) 13–17 (–18) mm long (spur excluded), 2-lipped, spurred, open at throat, violet; upper lip 2-lobed, dark violet, with lobes suborbicular to obovate, obtuse; lower lip 3-lobed, hairy throat, with lobes 5–10 mm long, longer than wide, obovate, overlapping, obtuse, coloured only in the distal half, the middle entire to subemarginate, white- to yellow-spotted near of the base; palate dark violet, except in the middle lobe of the lower lip; tube short, broadly infundibuliform, sparsely glandular outside; spur (6–) 7–11 (–13) mm long, cylindric-subulate, straight, sometimes slightly bifid. Stamens 2, epipetalous. Ovary superior, 1-locular; placenta free-central. Capsule 25–46 mm long, ovoid to subglobose, opening by 2 valves near the apex; seeds 0.5–0.6 mm long, numerous, ovoidal, reticulate. 2n = 48.

Pinguicula mundi G. Blanca, M. Jamilena, M. Ruiz-Rejón, and R. Zamora, **spec. nova** (Fig. 3).

A *P. vallisneriifolia* differt foliis quidem aestivis ellipticis vel obovatis, corolla (12–) 14–18 (–19) mm–huius autem calcar (8–) 9–12 (–13) mm atque faux intense violaceae praeterquam quoad inferioris labii lobulum centralem–, seminibus anguste ellipsoideis atque numero chromosomatum 2n = 48. Differt vero a *P. submediterranea* foliis suberectis, aestate non parce demum elongatis, corolla venis violaceis tenuibus itemque seminibus anguste ellipsoideis.

Holotypus: In orto fluminis Mundo, loco dicto Cueva de los Chorros, Sierra del Calar del Mundo, pr. Riópar (Albacete provincia, Hispania), 1200 m s.m. alt. ubi legit R. Zamora, 15-VI-1993 (GDAC 37729).

English description. Small insectivorous, scapose and perennial herbs, with slender roots and stolons present, overwintering as a bud. Leaves 6–12 (–15), in a basal rosette, suberect, entire, sessile, soft, and fleshy, with prominent median nerve on the back, clothed with viscid glands above, with the margin not involute, slightly undulate; spring leaves (anthesis) 3–6 × 1.5–2.5 cm, elliptic to obovate-oblong; summer leaves (fruiting) longer (6–10 × 1.5–3 cm), elliptic to obovate. Flowers solitary, zygomorphic; pedicels 1–10, 5–12 cm long, naked, covered with viscid glands, the stalks not more than twice as long as the gland. Calyx persistent, 2-lipped, with viscid glands; upper lip 3-lobed, with lobes 2.5–3.5 mm long, trian-

gular-obtuse or slightly elliptic, obtuse or subacute; lower lip 2-lobed, divided for up to 1/2 its length. Corolla (12–) 14–18 (–19) mm long (spur excluded), 2-lipped, spurred, open at throat, violet, sometimes with weakly marked veins; upper lip 2-lobed, dark violet, with lobes suborbicular to obovate, obtuse; lower lip 3-lobed, hairy throat, with lobes 6–12 mm long, longer than wide, obovate, overlapping, obtuse, coloured only in the distal half, the middle white- to yellow-spotted near of the base; palate dark violet, except in the middle lobe of the lower lip; tube short, broadly infundibuliform, sparsely glandular outside; spur (8–) 9–12 (–13) mm, cylindric-subulate, straight, sometimes slightly bifid. Stamens 2, epipetalous. Ovary superior, 1-locular; placenta free-central. Capsule 30–50 mm, ovoid to subglobose, opening by 2 valves near the apex; seeds 0.8–1 mm, numerous, narrowly ellipsoidal, reticulate. 2n = 48.

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