

Original Article

Evolutionary history of *Pinguicula* sect. *Pinguicula* (Lentibulariaceae)

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ABSTRACT

Although the main evolutionary lineages within *Pinguicula* have been identified, relationships at lower taxonomic levels remain unclear. In this study, we focused on *P. sect. Pinguicula*, the most species-rich group of butterworts in Europe, to identify key evolutionary lineages and explore potential speciation pathways. To achieve this, we applied cytological (karyology and flow cytometry) and molecular (AFLP and DNA sequencing) approaches. Contrary to literature records stating three ploidy levels, we found that *P. sect. Pinguicula* probably comprises taxa of only two ploidy levels—tetraploid ($2n=32$) and octoploid ($2n=64$). Despite the continuous variation in genome size between taxa, which can help in their delimitation, this trait lacks phylogenetic signal. Sequenced regions confirmed low sequence divergence, but the pattern of polymorphisms and duplications, together with AFLP data, allowed us to infer possible diversification routes. Hybridization and polyploidization within hybrid swarms appear to have played a crucial role in diversification. Tetraploid taxa and hybridization between them, followed by polyploidization and geographical isolation, probably set the wheel of speciation in motion. Pleistocene climatic oscillations further influenced this process by repeatedly expanding and contracting suitable habitats, facilitating additional hybridization and speciation events. Notably, octoploid taxa exhibit high genetic uniformity, suggesting they originated from a restricted gene pool.

Keywords: AFLP; biogeography; butterworts; carnivorous plants; chromosome counts; DNA sequencing; genome size

INTRODUCTION

The plant kingdom showcases a remarkable diversity of life strategies, with carnivory being one of the most intriguing. This unique adaptation has long captivated naturalists and scientists, including Charles Darwin. In his groundbreaking work *Insectivorous Plants* (Darwin 1875), Darwin was the first to suggest that carnivory evolved independently in multiple plant lineages. Over 150 years later, it is evident that this strategy, which enables plants to acquire essential nutrients in nutrient-deficient environments, has independently evolved at least 11 times across unrelated taxa (e.g. Albert *et al.* 1992, Ellison and Gotelli 2009, Fleck and Jobson 2023). The repeated emergence of

carnivory serves as a compelling example of convergent evolution, spanning genetic, metabolomic/physiological, and morphological levels (e.g. Fukushima *et al.* 2017, Hedrich and Fukushima 2021, Pavlovič *et al.* 2024). Among the families containing carnivorous plants, Lentibulariaceae Rich. stands out as the most species-rich, comprising three highly specialized genera: *Genlisea* Benth. & Hook.f., *Utricularia* L., and *Pinguicula* L., which together encompass ~385 species (Stevens 2001). This family exhibits an accelerated mutation rate in chloroplast DNA (cpDNA) coding sequences compared to other carnivorous plant genera and the broader angiosperm clade (Wicke *et al.* 2014, Fu *et al.* 2023).

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Pinguicula represents an early-diverging genus within the family Lentibulariaceae (e.g. Jobson and Albert 2002, Silva et al. 2016, Fleischmann and Rocca 2018). The split between the *Pinguicula* and the *Genlisea/Utricularia* clades has been estimated at 42 Mya (Bell et al. 2010). The subsequent diversification of butterworts probably occurred towards the end of the Tertiary, with estimates ranging from 16.4 Mya (Silva et al. 2018) to 9.87 Mya (Ibarra-Laclette et al. 2013). The genus is primarily distributed across the Northern Hemisphere, while its Southern Hemisphere range is restricted to the mountainous regions of western South America and its southernmost tip (Casper 1966, Rocca et al. 2016, Pérez et al. 2023). The genus has two major centres of diversity: one in the Americas (southeastern USA, Mexico, the Caribbean, Central and South America) and the other in Europe, particularly in the Mediterranean (Casper 1966, Rocca et al. 2016, Fleischmann and Rocca 2018). Hybridization and geographical isolation are considered the primary drivers of *Pinguicula* species diversity (Shimai et al. 2021, Majeský et al. 2022). Despite numerous efforts to resolve the phylogeny of the genus, only broad relationships have been clarified, while finer-scale relationships within species trees remain unresolved (e.g. Cieslak et al. 2005, Degtjareva et al. 2006, Shimai et al. 2021). The earlier infrageneric classifications of Casper (1962a, 1963, 1966) has been revised to better align with phylogenetic evidence, now recognizing three subgenera and 12 sections within the genus (Fleischmann and Rocca 2018, Fleischmann 2021).

In Europe, most species belong to *P.* subgenus *Pinguicula*, with a single section, *P.* sect. *Pinguicula* Casper, comprising ~30 species (Casper 1963, Fleischmann and Rocca 2018, Fleischmann 2021). This section is divided into three groups by chromosome number variation: diploids ($2n = 16$), tetraploids ($4n = 32$), and octoploids ($8n = 64$) (e.g. Casper and Stimper 2009). So far, only two diploid species are known, *P. corsica* Bernard & Gren. ex Gren. & Godr. and *P. schuensis* Bacch., Cannas & Peruzzi, which are considered relict and basal members of the subgenus (e.g. Casper 1966, Bacchetta et al. 2014, De Castro et al. 2016). Tetraploid species, accounting for one-third of the section's diversity, are thought to be of polytopic origin (Casper and Stimper 2009). In contrast, the octoploid taxa are considered to be evolutionarily young, having emerged during the Pleistocene (e.g. Casper 1966, Casper and Stimper 2009, Shimai et al. 2021). Apart from the ploidy level and geographical distribution, few morphological characters are useful for reliable species determination. Vegetative traits, such as leaf and rosette morphology, have limited diagnostic value and are mainly used to distinguish between isophyllous species (with uniformly sized leaves) and anisophyllous species (with leaves of varying sizes, where spring rosettes bear shorter leaves, and summer rosettes develop elongated leaves after flowering; Table 1). Most species in this section are found in the mountainous regions and canyons of the European Mediterranean and the Alps. Farther north, only members of the *P. vulgaris* L. group occur. The group comprises several morphologically similar octoploid taxa, of which *P. vulgaris* subsp. *vulgaris* is the most common octoploid species within the genus, reaching the widest geographical range (Casper 1966, Rocca et al. 2016, Fleischmann and Rocca 2018, Majeský et al. 2022). Other widespread species include the octoploid *P. macroceras* Link., which spans from Japan across the Kamchatka Peninsula to Alaska, Oregon, and California (Casper

1962b, 1966), and the tetraploid *P. grandiflora* Lam., which is distributed from the Alps, Jura Mountains, and Pyrenees to Ireland (Casper 1966, Rocca et al. 2016). Most of the other species within the section are geographically isolated, with restricted distributions.

This study focuses on the native European butterworts of *P.* subgenus *Pinguicula* sect. *Pinguicula*, aiming to clarify evolutionary relationships and outline diversification patterns within the group. Although some species have been included in broader phylogenetic analyses using nuclear and chloroplast loci, these studies have failed to resolve a stable phylogeny below the section level (Cieslak et al. 2005, Degtjareva et al. 2006, De Castro et al. 2016, Crespo et al. 2019, 2020, Shimai et al. 2021). The limited number of markers, whether nuclear or chloroplast, has led to unresolved polytomies and inconsistent results across studies, with phylogenetic relationships often appearing to be marker dependent (e.g. Shimai et al. 2021). To address this, we employed the AFLP technique, which has demonstrated superior resolution for inter-specific relationships (e.g. Majeský et al. 2022). By sequencing four cpDNA loci and one nuclear locus, we examined patterns of shared polymorphisms and duplications to infer relationships within *P.* sect. *Pinguicula* and to propose a diversification scenario. These molecular findings were complemented by karyological analysis and genome size estimates, providing a comprehensive view of the evolutionary dynamics of this group.

MATERIAL AND METHODS

Plant material

In this study, we adopted a population-based sampling approach, ensuring that, whenever possible, each taxon was represented by multiple samples from different localities. We examined 28 taxa belonging to *Pinguicula* sect. *Pinguicula*, covering over 80% of the species diversity within this section (Fig. 1; Table 1; Appendix 1). Additionally, *Pinguicula hirtiflora* Ten. (*P.* subgenus *Isoloba*, sect. *Cardiophyllum* Casper—as per Fleischmann 2021) was included as an outgroup. The analysed taxa were documented either through herbarium specimens deposited in the OL Herbarium (Department of Botany, Faculty of Science, Palacký University in Olomouc) or through photographic records. Abbreviations for taxa, consisting of three letters, are listed in Appendix 1.

Karyological analysis

Chromosome counts were conducted for species lacking prior data, those with contradictory reports, or where our genome size analysis suggested a different ploidy level. These species included *P. casperiana* M.B. Crespo, Mart.-Azorín, M.Á. Alonso & L. Sáez, *P. caussensis* (Casper) Rocca, Gluch, Lampard, A.S. Rob., A. Fleischm., S. McPherson, L. Legendre, Partrat & P. Temple, *P. christinae* Peruzzi & Gestri, *P. corsica*, *P. mariae* Casper, *P. mundi* Blanca, Jamilena, Ruíz Rejón & Reg. Zamora, *P. reichenbachiana* J. Schindl., *P. vallisneriifolia* Webb, and *P. vallis-regiae* F. Conti & Peruzzi. Root tip meristems from potted plants were used for karyological analysis. The root tips were pretreated with a 0.002 M aqueous solution of 8-hydroxyquinoline at 4°C for ~16 h (overnight), fixed in a 1:3 mixture of 98% acetic acid and 96% ethanol for 1–24 h, rinsed in distilled water, macerated in 1 N HCl at 60°C for 6 min, and washed again in distilled water. The squashes were prepared using the cellophane square technique

Table 1. Species list of currently recognized members of *Pinguicula* sect. *Pinguicula*.

Taxon	Intraspecific taxa	Geographical distribution	Rosette	Chromosome number	Ecology
4x taxa					
<i>P. balcanica</i> Casper	<i>*balcanica</i>	Balkan Peninsula (Dinaric Alps, Pindus Mts, Balkan Mts, Rhodope Mts)	ISO	32 ^{CS}	Growing on limestone, flysch, serpentine, granites; (900) 1500–2600 m
	<i>^apontica</i> Casper	TR: Taurus Mts, Pontic Mts; GE: Caucasus Mts	ISO	64 ^{CS}	n.a.
** <i>P. corsica</i> Bernard & Gren. ex Gren. & Godr.		Corsica	ISO	? 16 ^{CON, CS} , X32	Alkaline soils at low altitudes, acidic habitats at higher alt., 518–2200 m
<i>P. grandiflora</i> Lam.	<i>*grandiflora</i>	Widespread taxon, from Jura Mts through Pyrenees along Atlantic coast to Ireland	ISO	32 ^{DOU, CS}	Wide range from alkaline to acidic habitats, 0–2500 m
	<i>*rosea</i> (Mutel) Casper	FR., Isère, Savoie, Haute-Savoie depts.	ISO	32 ^{CON}	Wide range from alkaline to acidic habitats, 340–2000 m
<i>*P. leptoceras</i> Rchb.		Alps in SE France, NW Italy, S Switzerland, S and W Austria	ISO	32 ^{CON}	Acidic soils, 350–3005 m
<i>*P. longifolia</i> Ramond ex DC.		Pyrenees, Huesca province (ES), Hautes-Pyrénées (FR)	ANI	32 ^{ZA}	Calcareous, 500–2000 m
** <i>P. mariae</i> Casper		IT, S Apuan Alps	ISO	32 ^{CS2}	Alkaline substrates, calcareous cliffs, 250–1300 m
<i>*P. nevadensis</i> (H. Lindb.) Casper		ES, Sierra Nevada Mts	ISO	32 ^{CS}	High mountain wet meadows and bogs around lakes, 2500–3100 m
<i>*P. poldinii</i> J. Steiger & Casper		IT, Alps in N Italy	ISO	32 ^{CS2}	Calcareous, calcareous cliffs, 450–550 m
** <i>P. reichenbachiana</i> J. Schindl.		FR, Alpes Maritimes dept., IT—Savona prov.	ANI	32 ^{DOU}	Calcareous, calcareous cliffs, 150–800 (1500) m
** <i>P. vallisneriifolia</i> Webb		ES, Sierras de Cazorla, Segura y las Villas	ANI	32 ^{LK}	Calcareous, calcareous cliffs, 130–1700 m
8x taxa					
<i>*P. apuana</i> Casper & Ansaldo		IT, Apuan Alps, Apennine Mts	ISO	64 ^{CS}	Alkaline habitats, calcareous cliffs in Apuan Alps; slightly acidic pastures in Apennine, 200–1950 m
** <i>P. casperiana</i> M.B. Crespo, Mart.-Azorín, M.Á. Alonso & L. Sáez		ES, Cuenca and Guadalajara prov.	sANI	64 ^{CS}	Limestone cliffs, 850–1550 m
** <i>P. caussensis</i> (Casper) Rocca, Gluch, Lampard, A.S. Rob., A. Fleischm., S. McPherson, L. Legendre, Partrat & P. Temple		FR, Massif Central, gorges of les Grandes Causses	sANI	? 32 ^{CS} , X64	Calcareous species, limestone cliffs of gorges; 300–900 m
** <i>P. christinae</i> Peruzzi & Gestri		IT, N Apennines	ISO	64 ^{CS2}	Acidic habitats on ophiolitic soils, 1000–1850 m
** <i>P. dertosensis</i> (Cañig.) Mateo & M.B. Crespo		SP, Puertos de Beceite y Torosa Mts	sANI	64 ^{CS}	Calcareous species of various habitats, 350–1750 m
<i>*P. fiorii</i> Tammara & L. Pace		IT, Apennine Mts, Majella massif	ISO	64 ^{CS, M}	Calcareous cliffs, 750–2400 m
<i>*P. macroceras</i> Link		Amphi-Pacific distribution, from Japan and Kamchatka through Alaska across W Canada down along the western coast to Oregon and California, widespread	ISO	64 ^{U, CS}	Acidic substrates, 0–2500 m
** <i>P. mundi</i> Blanca, Jamilena, Ruiz Rejón & Reg. Zamora		ES, Albacete province	ANI	64 ^{CS}	Calcareous, calcareous cliffs, 1000–1250 m

(Continued)

Table 1. Continued.

Taxon	Infraspecific taxa	Geographical distribution	Rosette	Chromosome number	Ecology
** <i>P. vallis-regiae</i> F. Conti & Peruzzi		IT, Abruzzo, L'Aquila prov.	ISO	64 ^c	Calcareous cliffs, 1073 m
<i>P. vulgaris</i> L.	<i>*vulgaris</i>	Widespread across the temperate parts of the entire N hemisphere	ISO	64 ^{CS}	From acidic to alkaline substrates and habitats, 0–2600 m
	<i>*anzalonei</i> Peruzzi & Conti	IT, central Italy	ISO	64 ^M	Limestone cliffs, 700 m
	<i>*bohémica</i> (Kraj.) Domin	CZ, central Bohemia	ISO	64 ^{KJ,M}	Calcareous fens, lowlands, 180–260 m
	<i>*ernica</i> Peruzzi & Conti	IT, central Italy	ISO	64 ^c	Limestone cliffs, 700 m
	<i>*vestina</i> Conti & Peruzzi	IT, central Italy	ISO	64 ^c	Calcareous substrates, above 1000 m
Taxa of unknown ploidy					
^b <i>P. arvetii</i> P.A. Genty		FR: Alpes Maritimes, Alpes de Haute Provence, Hautes Alpes depts.; IT: Cuneo and Torino provs.	ISO	Supp. 4x/6x	Acidic soils, calcareous seepages; 1800–3000 m
^c <i>P. fontiqueriana</i> Romo, Peris & Stübing		MA, Rif Mts	ISO	Supp. 8x	Bogs and streams in cedar forests, 1700–2100 m
<i>P. lattaniziae</i> Peruzzi & Gestri		IT, N Apennines, Monte Aiona	?	Supp. 8x	1600–1700 m
<i>P. saetabensis</i> M.B. Crespo, Mart.-Azorín & M.Á. Alonso		ES, Saetabensian Mts, Valencia prov.	ANI	Supp. 8x	Calcareous cliffs, 400–600 m
<i>P. sehuensis</i> Bacch., Cannas & Peruzzi		IT, Sardinia	ISO	? 16 ^b , supp. 4x	Calicolous, calcareous cliffs, 1035–1185 m
<i>P. submediterranea</i> Blanca, Jamilena, Ruíz-Rejón & Reg. Zamora		ES, Sierra de Cazorla, Jaén prov.	ISO	Supp. 8x	Calcareous soils around streams and springs
<i>P. tejedensis</i> M.B. Crespo, M. Azorín, M.Á. Alonso & L. Sáez		ES, Sierras de Tejeda, Granada prov., Betic Mts	sANI	Supp. 8x	Dolomite cliffs, 1000–1800 m
Natural hybrids encountered in sympatric regions of paternal species					
<i>P. caussensis</i> × <i>grandiflora</i> subsp. <i>grandiflora</i>		FR, Aveyron dept.	ISO	?	Alkaline habitats, 800 m
<i>P. grandiflora</i> subsp. <i>grandiflora</i> × <i>longifolia</i>		ES, Huesca prov.; FR: Hautes Pyrénées dept.	ANI	?	Alkaline substrate, calcareous cliffs, 1000–1700 m
<i>P. reichenbachiana</i> × <i>vulgaris</i>		FR, Alpes Maritimes dept.	?	?	Alkaline habitats, 420 m
<i>P. grandiflora</i> subsp. <i>rosea</i> × <i>vulgaris</i> [<i>Pinguicula</i> × <i>scullyi</i> nothosubsp. <i>gresivaudanica</i> (Roccia) J. Schlauer]		FR, Isère, Savoie, Haute-Savoie depts.	ISO	48 ^R	Alkaline habitats, 880–1540 m
<i>P. grandiflora</i> subsp. <i>grandiflora</i> × <i>vulgaris</i> (<i>P.</i> × <i>scullyi</i> Druce)		IL, Pyrenees, Andorra, French Alps, French Jura, etc	ISO	?	From alkaline to acidic soils, 300–2200 m
<i>P. vulgaris</i> subsp. <i>vulgaris</i> × <i>vulgaris</i> subsp. <i>bohémica</i> (<i>P. vulgaris</i> nothosubsp. <i>dostalii</i> (Bárta) ined.)		CZ, central Bohemia	ISO	64 ^{KJ}	Calcareous fens, 180 m

Within many of the species listed, striking morphological deviations from the typical morphotype are recognized at the level of 'forma' or 'variety'. These oddities seem to have a genetic background of simple mutations and are not included in the table. Taxa used in the present study are marked with '**' and those studied karyologically are marked with '***'. The column 'rosette' refers to three recognized growth types: ISO—isophylly (leaves of spring and summer rosette of equal length); ANI—anisophylly (leaves of summer rosette greatly enlarged); sANI—slight anisophylly (leaves of summer rosette only slightly enlarged). Only verified chromosome numbers are given in the table with reference to the original literature (see footnote); ?—chromosome reports considered doubtful by us; X—a different number of chromosomes than traditionally reported was observed in the present study; supp.—supposed ploidy level. depts, departments.

^a*P. balcanica* subsp. *pontica* may represent octoploid *P. vulgaris* instead of a tetraploid taxon.

^bTaxonomic status of *P. arvetii* is not resolved nor stable, e.g. an aberration of *P. leptoceras* (Casper 1966); hybrid *P. vulgaris* × *P. leptoceras* (Roccia et al. 2016).

^c*P. fontiqueriana* represents another doubtful species, and may rather represent the southernmost occurrence of *P. vulgaris* (Roccia et al. 2016).

B, Bacchetta et al. 2014; CON, Contandriopoulos 1962; C, Caparelli et al. 2008; CS2, Casper and Stimpert 2006; CS, Casper and Stimpert 2009; DOU, Doulat 1947; KJ, Krahulcova and Jarolimová 1991; LK, Löve and Kjellqvist 1974; M, Majesky et al. 2022; R, Roccia 2013; U, Uchiyama 1990; ZA, Zamora et al. 1996.

(Murin 1960). Slides were stained with a 7% Giemsa solution (Sigma-Aldrich) in Sörensen phosphate buffer (pH 7.0), dried, and examined under a Leica DM 1000 microscope equipped with an

HDCE-X5 camera and ScopeImage 9.0 software in immersion oil. At least three replicates were made for each investigated species, and chromosomes were counted in 5–25 different cells.

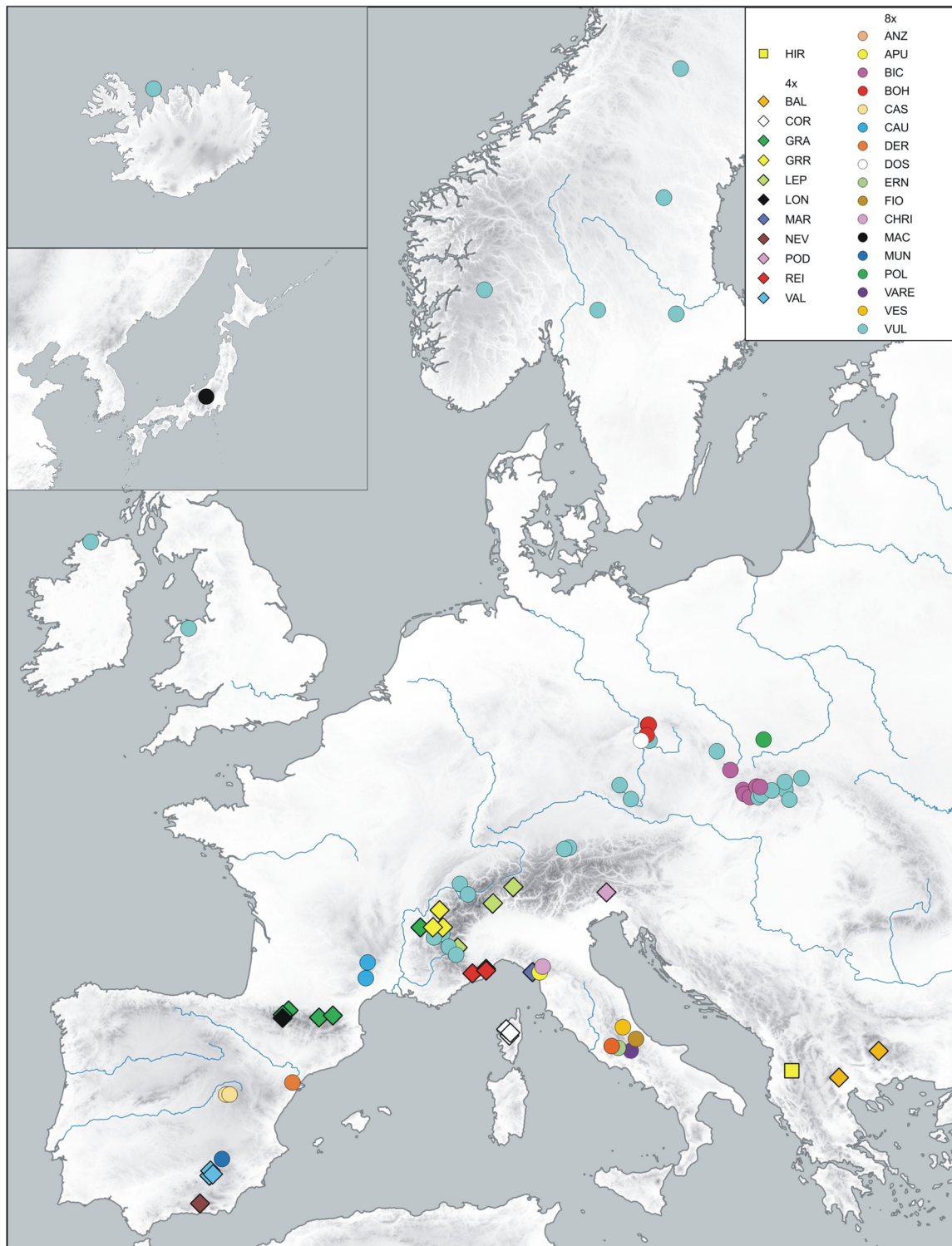


Figure 1. Map showing the geographical localization of the sampled taxa. *Pinguicula hirtiflora* was used as an outgroup. See Appendix 1 for three-letter taxon abbreviations.

Genome size measurements

The absolute genome size (AGS) of 134 individuals representing 28 taxa (Appendix 1) was determined using either a BD Accuri C6 flow cytometer (BD Biosciences, San Jose, CA, USA) equipped with a blue laser (488 nm, 20 mW, BD Accuri™; BD Biosciences) or a Partec PAS flow cytometer (Partec GmbH, Münster, Germany)

equipped with a diode-pumped solid-state green laser (532 nm, 100 mW, Cobolt Samba; Cobolt AB, Stockholm, Sweden). Sample preparation followed the protocol outlined by Doležal *et al.* (2007) and further detailed by Kobrová and Hroneš (2019).

Before analysis, samples were incubated at 4°C for ~4 h with occasional shaking. Fluorescence intensity was recorded for at

least 5000 particles per sample using the flow cytometer. *Glycine max* (L.) Merr. 'Polanka' ($2C = 2.50$ pg, Doležel *et al.*, 1994) was used as the primary internal reference standard, and on certain occasions, *Zea mays* L. CE-777' ($2C = 5.67$ pg, recalculated against *G. max*) served as a secondary internal standard. Histograms were accepted only if the coefficients of variation (CV) for the G0/G1 peaks of both the sample and standard were below 5%. Each individual was analysed at least three times, with the average value used to calculate AGS. If the absolute differences among the three measurements exceeded 5%, the most outlying measurement was excluded, and a new measurement was performed. Within-sample variation was calculated as [(maximum value – minimum value)/average value of all three measurements] $\times 100$. The monoploid genome size (1Cx value, Greilhuber *et al.* 2005) was calculated by dividing the $2C$ value by the ploidy level, as determined from published chromosome counts or counts determined within this study. The 1Cx values were correlated with the geographical coordinates (longitude and latitude) of the respective populations. DNA-ploidy levels were inferred from AGS values following Suda *et al.* (2006). Differences in AGS and 1Cx values among taxa were not statistically tested due to uneven sample sizes. Descriptive statistics and correlation analyses were performed using NCSS 9 (Hintze 2013).

DNA extraction and AFLP fingerprinting

Genomic DNA was extracted from fresh leaves of 205 individuals representing 29 taxa (Appendix 1) using the protocol of Doyle and Doyle (1987), with minor personal modifications. AFLP analysis was conducted using three selected primer combinations (Table S1), following the method of Vos *et al.* (1995) with modifications described by Ali *et al.* (2017). Amplified fragments were separated using a GenomeLab CEQ/GeXP DNA analyser (Beckman Coulter, USA), and raw data were processed with CEQ 8000 software. Peaks within the size range of 70–600 bp were scored as present (1) or absent (0). To ensure reproducibility, the analysis included quality controls such as blind samples, duplicate samples, and repeated runs.

AFLP data analysis

Due to uneven sampling, population- or taxon-based statistics were not calculated from the AFLP data. Instead, the binary AFLP data were used for clustering analyses employing various approaches, and the results were compared to assess data robustness. A matrix of pairwise genetic distances based on the Jaccard similarity coefficient was used in neighbour-joining analysis to construct a dendrogram. The dendrogram's support was evaluated with 1000 bootstrap replications. All computations were performed in DARwin 6.0.21 (Perrier and Jacquemoud-Collet 2006), and the resulting dendrogram was visualized in FIGTREE (<http://tree.bio.ed.ac.uk/software/figtree/>), with *P. hirtiflora* designated as the root.

Bayesian clustering, implemented in STRUCTURE (Falush *et al.* 2007), was performed using the recessive allele model and the admixture model with correlated allele frequencies. The number of clusters (K) was set from 1 to 26, with 10 replicate runs for each K , using 1000000 Markov chain Monte Carlo (MCMC) iterations following a burn-in period of 100000

iterations. Results were summarized using Structure Harvester (Earl and von Holdt 2012), CLUMPP (Jakobsson and Rosenberg 2007), and DISTRUCT (Rosenberg 2004). Based on the weak sequence differentiation of the two *P. grandiflora* subspecies, the limited geographical origin of the investigated samples and trial runs of the analysis, *P. grandiflora* was treated as a single taxon in the STRUCTURE analysis. Principal coordinate analysis (PCoA), based on a Jaccard similarity matrix, was performed using FAMD (Schluter and Harris 2006).

Sequencing, sequence alignment, and phylogenetic analysis

A total of 130 samples representing 29 taxa were sequenced for four chloroplast loci (*trnT-trnF*, *petN-psbM*, *trnQ-rps16*, and *trnK-matK*) and one nuclear locus (ITS1–5.8S rDNA–ITS2) (Appendix 1). PCRs were conducted in a 25- μ L volume containing 20 ng of template DNA, 2 μ M of each primer, 0.2 mM dNTPs, 1 $\times$ PCR reaction buffer (with 2 mM $MgCl_2$), and 1 U of GoTaq[®] DNA polymerase (Promega Corp., USA). The reaction conditions were as follows: initial denaturation at 95°C for 3 min; 36 cycles of denaturation at 95°C for 30 s, annealing at an experimentally determined temperature (Table S2) for 45 s, and extension at 72°C for 1 min; followed by a final extension at 72°C for 10 min. PCR products were purified using the GeneElute PCR Clean-Up Kit (Sigma-Aldrich) and sequenced bidirectionally by MacroGen Europe (Amsterdam, The Netherlands). Sequences were edited and aligned using the MAFFT algorithm in Geneious[®] 7.1.7 (Kearse *et al.* 2012, <http://www.geneious.com>). The sequence data have been deposited in GenBank (accession numbers available in Table S3). To ensure accuracy and verify against possible misidentification or technical errors, available GenBank records for the ITS and *trnK-matK* loci were compared with our samples (see Table S4 for the list of compared records). Phylogenetic analyses were performed in MrBayes (Ronquist *et al.* 2012) separately for ITS (GTR+G+I nucleotide substitution model, two simultaneous runs of four parallel Metropolis-coupled MCMC (MCMCMC) chains, each for 1 100 000 generations, with a random starting tree, and 25% of the trees discarded as burn-in) and chloroplast loci (GTR+G+I nucleotide substitution model, two simultaneous runs of four parallel MCMCMC chains, each for 1 700 000 generations, with a random starting tree, and 25% of the trees discarded as burn-in). Selection of the nucleotide substitution model was made using the module for nucleotide substitution model selection implemented in MEGA 11 (Tamura *et al.* 2021). The model with the lowest corrected Akaike information criterion (AICc) was chosen. For the ITS phylogeny, reliable GenBank records were included alongside our samples.

Since the phylogenetic analyses did not significantly refine previously reported relationships (e.g. de Castro *et al.* 2016), observed sequence polymorphisms from the ITS locus were converted into binary data, merged with AFLP data, and analysed using neighbour-joining analysis with Jaccard similarity coefficient. Only single-nucleotide polymorphisms (SNPs) with two nucleotide variants at a given position were binary coded (0 = common state, 1 = SNP variant). SNPs with three or more variants were excluded. Pairwise genetic distances (p-distances) and variance estimates (1000 bootstrap replications) were calculated in MEGA 11, and heatmaps were produced in the R package 'pheatmap' (Kolde 2018).

RESULTS

Karyology

Although butterworts are notoriously challenging for karyological analysis, we successfully counted chromosomes in ten taxa. Two cytotypes were identified: tetraploid ($2n=4x=32$) and octoploid ($2n=8x=64$). Four taxa (*P. corsica*, *P. mariae*, *P. reichenbachiana*, and *P. vallisneriifolia*) were determined to be tetraploid (Fig. 2), while the remaining six taxa [*P. casperiana*, *P. caussensis*, *P. christinae*, *P. dertosensis* (Cañig.) Mateo & M.B. Crespo, *P. mundi*, and *P. vallis-regiae*] exhibited the octoploid cytotype (Figs 3 and 4). The chromosome count for *P. vallis-regiae* represents the first record for this species. Notably, *P. corsica* was identified as tetraploid, and *P. caussensis* as octoploid, despite previous records establishing these species as diploid and tetraploid, respectively. These findings mark the first reports of these ploidy levels for both species.

Genome size

Together with our previous study (Majeský *et al.* 2022), we present genome size estimates for 28 taxa, for nine of which this is the first report. CVs for the standard and sample ranged from 0.96 to 3.93 (mean $\pm$ SD = 2.07 ± 0.56) and 1.29 to 4.94 (2.80 ± 0.77), respectively. Among the analysed species, AGS varied 3.28-fold, with an average of 1.26 ± 0.33 pg. The lowest AGS value was 0.60 pg in *P. nevadensis* (H. Lindb.) Casper, while the largest AGS was 1.97 pg in *P. vallis-regiae*. The monoploid genome size (1Cx value) ranged from 0.15 pg in *P. nevadensis* to 0.26 pg in *P. grandiflora* subsp. *grandiflora* (0.20 ± 0.03). Flow cytometry measurements identified two AGS groups, broadly corresponding to DNA-tetraploids and DNA-octoploids. However, AGS variation within a single ploidy level was substantial, reaching 57% and 45% for tetraploids and octoploids, respectively. The AGS values showed no phylogenetic signal, i.e. sister species showed significant differences in their 1Cx values (not statistically tested), but most taxa on the same ploidy level were distinguishable from each other by their AGS values (Table 2; Fig. 5). Nevertheless, a slight trend of genome downsizing was observed towards terminal groups (Fig. 5B). Additionally, 1Cx values were rather weakly negatively correlated with latitude (Pearson $r = -0.3680$, $P = 0.0001$; Fig. S1A), reflecting the distribution of taxa from terminal groups in higher latitudes. A correlation between 1Cx and longitude was very weak and not statistically significant (Pearson $r = -0.1491$, $P = 0.1253$; Fig. S1B).

AFLP data

A total of 520 clearly scorable markers were obtained from the three AFLP-primer combinations, with a 3% error rate. The neighbour-joining clustering analysis resulted in a dendrogram where most samples formed taxon-specific clusters (Fig. S2). Only *P. christinae* appeared in two separate clusters. In the dendrogram, tetraploid taxa (4x) were separated from octoploid taxa (8x), with 4x taxa forming the first diverging clades. Notably, *P. caussensis*, previously thought to be tetraploid, was placed within the octoploid clade, alongside *P. vulgaris* group. Among the 4x taxa, *P. leptoceras* Rchb. was the first to diverge, followed by clusters of *P. mariae* and *P. reichenbachiana*. *Pinguicula poldinii* J. Steiger & Casper came next, followed by a group of *P. corsica*, *P. nevadensis*,

P. balcanica Casper, *P. longifolia* Ramond ex DC., and *P. vallisneriifolia*. Here, *P. corsica* and *P. nevadensis* clustered together as a sister clade to *P. balcanica*, while *P. longifolia* and *P. vallisneriifolia* were basal. The large cluster of *P. grandiflora* was placed as the sister branch to all the octoploid taxa (Fig. S2). The two subspecies of *P. grandiflora* [subsp. *grandiflora* and subsp. *rosea* (Mutel) Casper] formed separate clusters, with one sample of subsp. *grandiflora* (GRA_696-7) clustering with subsp. *rosea*. In the octoploid group, *P. christinae* clustered with *P. vulgaris* subsp. *vestina* Conti & Peruzzi, followed by a distinct cluster of Iberian octoploid taxa. Here, *P. casperiana* formed a sister branch to the *P. dertosensis*–*P. mundi* cluster. *Pinguicula macroceras* and *P. fiorii* Tammaro & L. Pace formed another cluster. The central Apennine taxa, including *P. apuana* Casper & Ansaldo, *P. vallis-regiae*, and *P. christinae*, formed a separate group, while *P. caussensis* was placed as a sister branch to the *P. vulgaris* agg. cluster. The distinct taxa within *P. vulgaris* agg. formed separate clusters.

PCoA showed that the 4x taxa formed a more diverse, relaxed cluster, while the 8x taxa were more tightly grouped, particularly within *P. vulgaris* agg. (Fig. 6). *Pinguicula mundi* and *P. dertosensis* were placed between the 4x and 8x clusters. The first three axes explain 45% of the total variation.

The STRUCTURE analysis was straightforward, and Evanno's DeltaK method indicated the presence of two distinct clusters ($K=2$) as the best differentiation for the examined data. The presence of the two clusters within the studied taxa gradually changes from the 4x (dominant cluster 1: *balcanica*–*longifolia*–*vallisneriifolia*–*grandiflora*–*nevadensis*–*corsica*–*poldinii*–*mariae*–*leptoceras*–*reichenbachiana*) to the 8x taxa (dominant cluster 2: *christinae*–*vallis-regiae*–*apuana*–*caussensis*–*vulgaris* agg.) among which are the rest of the 8x taxa (*mundi*–*dertosensis*–*casperiana*–*macroceras*–*fiorii*–*vestina*) with nearly 50/50 representation of both clusters (Fig. 7A). Although the decision on the correct K was clear, the clustering for $K=4$ and 15 was also examined (Fig. 7A), based on the values of SD of $\text{LnP}(K)$ and DeltaK. For $K=4$ in the 4x taxa, the additional genetic clusters distinguished the *balcanica*–*grandiflora* from the *poldinii*–*mariae*–*leptoceras*–*reichenbachiana* group while the *nevadensis*–*corsica*–*longifolia*–*vallisneriifolia* group contains signal from both these genetic clusters. The 8x taxa represent mixed parts of the four genetic clusters, except the trio *bohemicabicolor*–*vulgaris*. For $K=15$, the 15 genetic groups are present in varying proportions in the investigated taxa, allowing a description of finer-grained relationships among the examined taxa (Fig. 7A).

DNA sequence data

All five sequenced regions showed very low variability, with only 164 parsimony-informative positions in the 5141-bp-long concatenated alignment (Table 3). The most variable was the nuclear ITS locus. Nucleotide diversity was low, ranging from 0.019 for ITS to 0.001/0.002 for the cpDNA loci (Table 3). Comparison of ITS sequences with GenBank records confirmed the correct taxonomic identification of all samples, eliminating the possibility of gross taxonomic misidentification. Most taxa had high intra-specific sequence homogeneity, with ribotype variants differing by only a few SNPs (Table S5). The only exception was *P. vallisneriifolia*, where two samples differed by 33 SNPs. *Pinguicula*

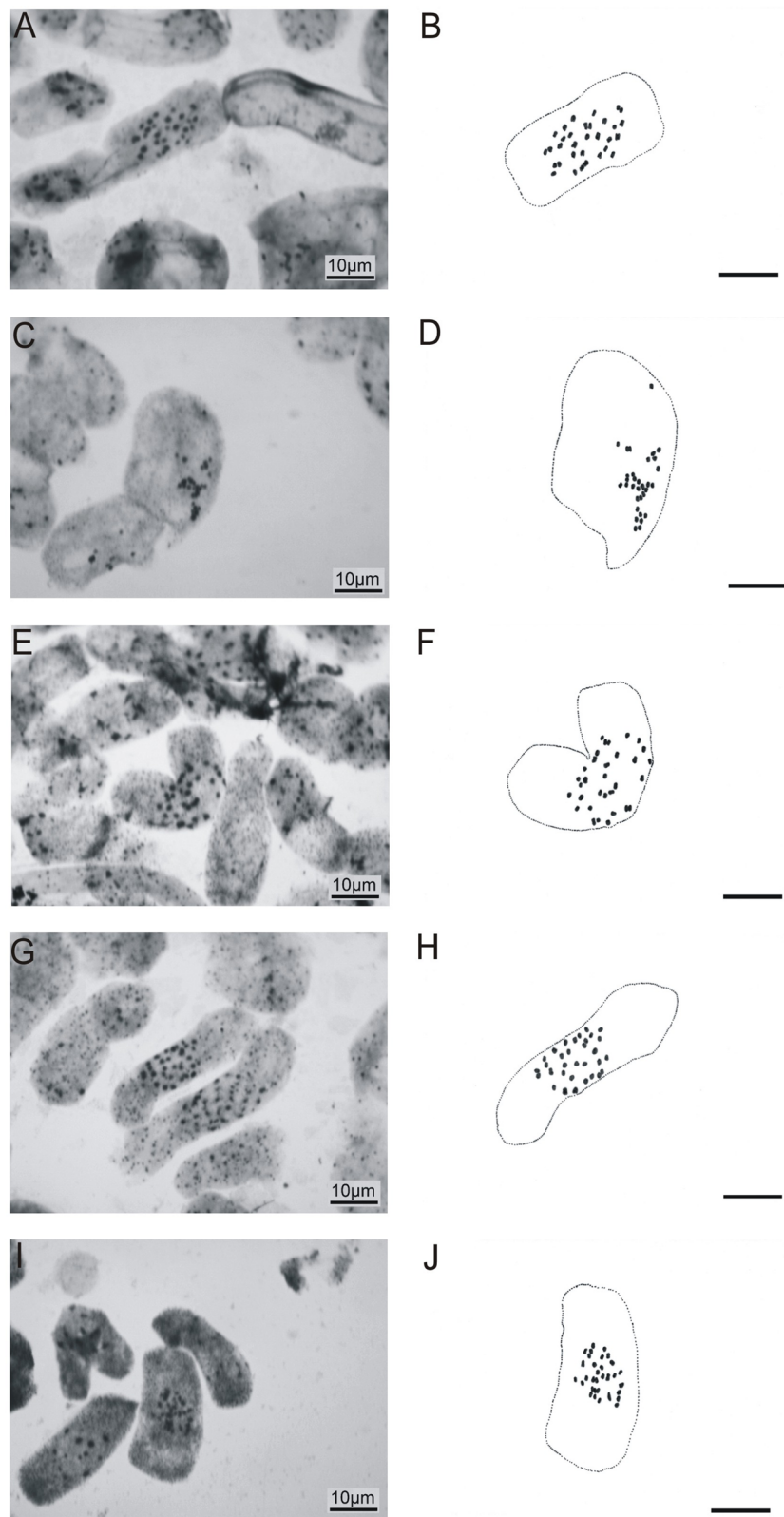


Figure 2. Mitotic metaphase plates of four tetraploid taxa of *Pinguicula* showing $2n = 32$ chromosomes. A–D, *P. corsica* (COR_477: A/B, COR_1: C/D); E–F, *P. mariae* (MAR_1); G–H, *P. reichenbachiana* (FON_1); I–J, *P. vallisneriifolia* (VAL_1).

grandiflora subsp. *grandiflora* and subsp. *rosea* differed by only three SNPs (116: A→G; 118: G→T; 602: C→T; Table S5). Interestingly, one sample of *P. grandiflora* subsp. *grandiflora* (GRA_696-7) clustered with *P. grandiflora* subsp. *rosea* (Fig. 7B; Fig. S2).

Both specimens come from the Isère region (France) where these two subspecies are sympatric. This suggests that the regional distribution, rather than taxonomic identity, influenced the clustering.

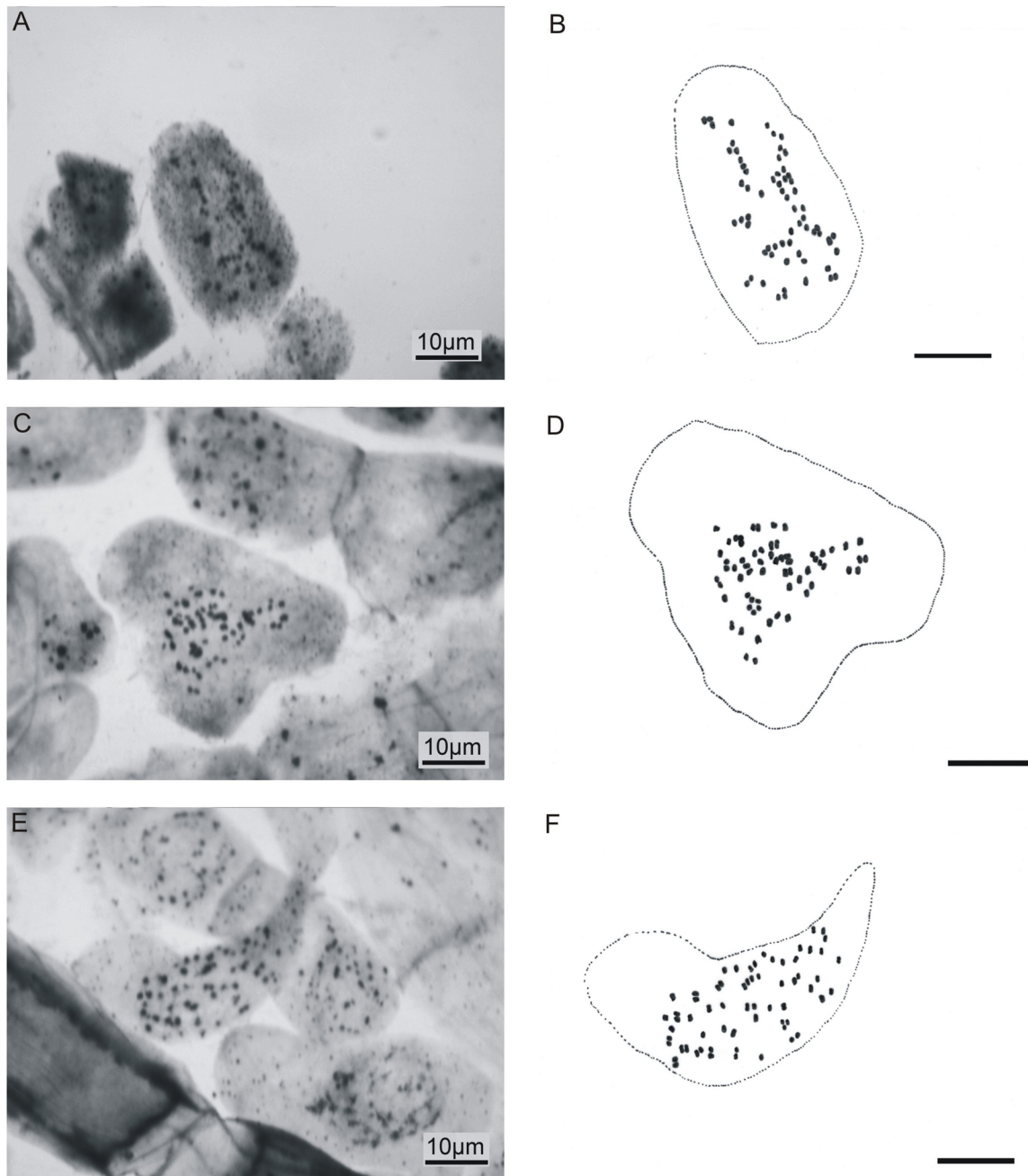


Figure 3. Mitotic metaphase plates of three octoploid taxa of *Pinguicula* showing $2n = 64$ chromosomes. A–B, *P. casperiana* (CAS_1373_6); C–D, *P. caussensis* (CAU_1335_9); E–F, *P. christinae* (CHR_1).

The sequenced cpDNA loci were less informative, showing low variability (Table 3; Table S5). However, some taxa showed noticeable length variation in these regions, mainly due to duplicated motifs. The pattern of these duplications corresponds well with relationships suggested by AFLP analysis. Some duplications were taxon-specific, such as a 6-bp motif (CTAATG) in *petN-trnC* and a 32-bp motif in *trnT-trnF* specific to *P. corsica*, and an 8-bp motif (AAATTAAA) in *trnQ-rps16* specific to *P. poldinii* (Table 4; Table S5). Low sequence divergence in the cpDNA prevented the construction of a well-supported phylogenetic tree, leading to unresolved polytomies. The 'ITS phylogenetic tree' (Fig. S3) was largely consistent with

previous studies, while the 'cpDNA tree' (Fig. S4) was even less informative. Merging AFLP data and ITS SNPs produced a more informative dendrogram, which was similar to the AFLP-based tree, with a few exceptions in the clustering of the 4x taxa (Fig. 7B). In this tree, *P. balcanica* formed the basal branch, followed by the 4x taxa, which were divided into two clusters: one with *P. longifolia*, *P. vallisneriifolia*, *P. nevadensis*, *P. corsica*, and *P. grandiflora* and another with *P. poldinii* as the basal branch to *P. mariae*, *P. leptoceras*, and *P. reichenbachiana*. The clustering of the 8x taxa showed better alignment with the AFLP tree, except for the three Iberian taxa (*P. mundi*, *P. dertosensis*, *P. casperiana*), which diverged early within the 8x group (Fig. 7B vs. Fig. S2).

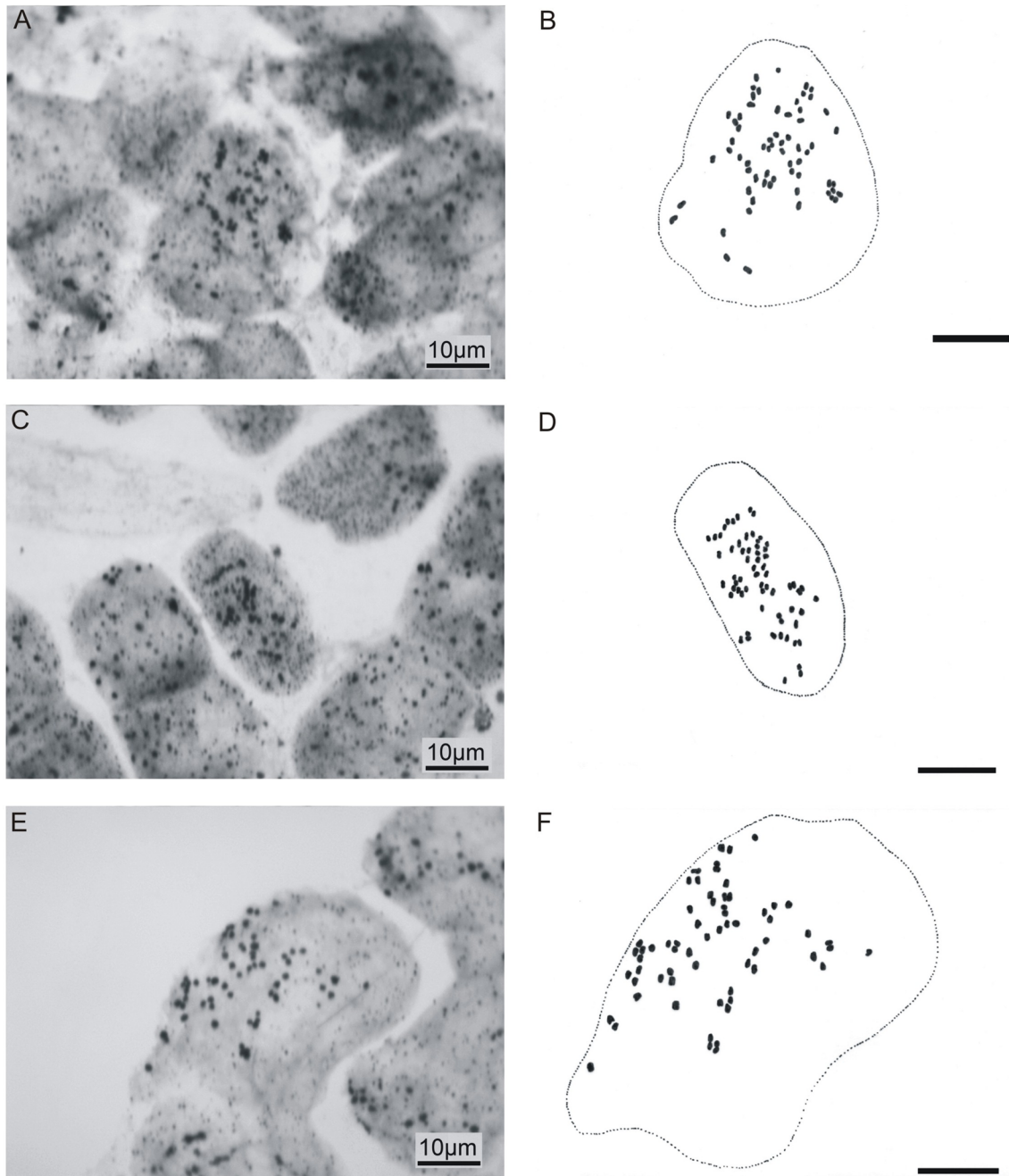


Figure 4. Mitotic metaphase plates of three octoploid taxa of *Pinguicula* showing $2n = 64$ chromosomes. A–B, *P. dertosensis* (DER_684_10); C–D, *P. mundi* (MUN_1600_4); E–F, *P. vallis-regiae* (VAR_2).

Heatmaps of pairwise p-distances (Fig. 7C, D) and the pattern of shared polymorphic sites (Table S5) provided further insight into the relationships. The 4x taxa *P. leptoceras*, *P. reichenbachiana*, and *P. mariae* showed minimal differentiation from most of the octoploid taxa in the nuclear ITS region (Fig. 7C) but were the most divergent at the cpDNA loci (Fig. 7D; Table S5). In other words, this group of 4x taxa (i.e. *P. leptoceras*, *P. mariae*, *P. reichenbachiana*) was more similar to all the 8x taxa (except *P. mundi*) at the nuclear ITS locus than the rest of the 4x taxa (i.e. *P. poldinii*, *P. balcanica*, *P. corsica*, *P. nevadensis*, *P. grandiflora* subsp. *grandiflora*, *P. grandiflora* subsp. *rosea*, *P. longifolia*, *P.*

vallisneriifolia). In contrast, the cpDNA sequences of most tetraploid taxa, including *P. balcanica*, *P. corsica*, *P. nevadensis*, *P. grandiflora* subsp. *grandiflora*, *P. grandiflora* subsp. *rosea*, *P. longifolia*, and *P. vallisneriifolia* exhibited a higher degree of similarity to the octoploid taxa, while sequences from *P. mariae*, *P. reichenbachiana*, *P. leptoceras*, and *P. poldinii* were distinct. The three Iberian taxa (*P. casperiana*, *P. dertosensis*, *P. mundi*) shared identical haplotypes but differed in ribotype, with *P. mundi* sharing a ribotype with *P. corsica* and *P. nevadensis* (Fig. 7C, D; Table S5). *Pinguicula caussensis* shared ribotype/haplotypes with *P. vulgaris* agg.

Table 2. Summary of flow cytometric genome size (AGS) estimation in analysed taxa sorted by their mean AGS.

Taxon	N	Mean AGS (pg)	±SD	Min–max (pg)	1Cx (pg)	Inferred ploidy level
<i>nevadensis</i> ^a	1	0.60	n.a.	n.a.	0.15	4
<i>vallisneriifolia</i>	7	0.73	0.020	0.70–0.76	0.18	4
<i>balcanica</i> ^a	4	0.76	0.020	0.73–0.78	0.19	4
<i>leptoceras</i> ^a	4	0.75	0.010	0.74–0.76	0.19	4
<i>poldinii</i> ^a	3	0.80	0.020	0.78–0.82	0.20	4
<i>corsica</i>	7	0.85	0.030	0.81–0.90	0.21	4
<i>longifolia</i> ^a	1	0.90	n.a.	n.a.	0.23	4
<i>rosea</i> ^a	3	0.95	0.030	0.92–0.97	0.24	4
<i>reichenbachiana</i>	9	0.96	0.070	0.87–1.06	0.24	4
<i>mariae</i> ^a	6	1.00	0.060	0.96–1.09	0.25	4
<i>grandiflora</i>	5	1.04	0.040	1.01–1.09	0.26	4
<i>ernica</i> ^b	4	1.32	0.010	1.31–1.33	0.17	8
<i>macroceras</i>	1	1.34	n.a.	n.a.	0.17	8
<i>bohemia</i> ^b	11	1.35	0.030	1.31–1.39	0.17	8
<i>anzaloni</i> ^b	3	1.36	0.030	1.34–1.39	0.17	8
<i>polonica</i> ^b	2	1.38	0.010	1.37–1.38	0.17	8
<i>x dostalii</i> ^b	2	1.38	0.002	1.376–1.379	0.17	8
<i>mundi</i>	3	1.43	0.030	1.37–1.46	0.18	8
<i>vestina</i> ^b	1	1.40	n.a.	n.a.	0.18	8
<i>casperiana</i> ^a	3	1.43	0.050	1.37–1.47	0.18	8
<i>vulgaris</i> ^{b,d}	33	1.44	0.070	1.35–1.61	0.18	8
<i>bicolor</i> ^b	3	1.46	0.020	1.44–1.47	0.18	8
<i>caussensis</i>	3	1.46	0.010	1.45–1.47	0.18	8
<i>dertosensis</i>	3	1.59	0.020	1.57–1.61	0.20	8
<i>apuana</i> ^{b,c}	3	1.72	0.040	1.68–1.74	0.22	8
<i>christinae</i> ^b	4	1.76	0.050	1.71–1.83	0.22	8
<i>fiorii</i> ^b	3	1.93	0.030	1.91–1.96	0.24	8
<i>vallis-regiae</i> ^a	3	1.97	0.010	1.96–1.97	0.25	8

^aRepresent the first reports of genome size for particular taxa.^bValues already reported by Majesky *et al.* (2022).^cDifferences are due to different gating method of respective histograms.^dDifferences are due to addition of the new samples.

1Cx, monoploid genome size, i.e. AGS divided by ploidy level; inferred ploidy level, ploidy level derived from AGS value; mean AGS, mean genome size of DNA; N, number of analysed individuals; n.a., not counted due to low sample size; SD, standard deviation of mean (in pg of DNA).

DISCUSSION

Cytological observations

Building on previous studies (Zamora *et al.* 1996, Casper and Stimper 2006, 2009), our research provides a comprehensive overview of karyological and genome size variation across nearly all recognized members of *Pinguicula* sect. *Pinguicula*. Consistent with earlier reports (Casper and Stimper 2006, 2009), we confirmed the existence of two main cytotypes, namely tetraploid ($2n=32$) and octoploid ($2n=64$). No evidence of diploids or other rare cytotypes was found. Notably, *P. corsica*, previously assumed to be diploid, was identified as a tetraploid (see below). The reliability of our findings is ensured by the use of plants from verified sources and sequence comparisons, minimizing the risk of taxonomic misidentification and discrepancies in ploidy assessments.

We provide the first karyological record for *P. vallis-regiae*, identifying it as an octoploid species ($2n=64$), in agreement with molecular analyses (Fig. 7). A chromosome count of $2n=64$ was also recorded for *P. caussensis*, which contrasts with the study by Casper and Stimper (2009), who reported this species as tetraploid but did not provide image documentation of this count. Additionally, we confirm the chromosome number

of $2n=64$ for *P. dertosensis* and *P. mundi*, aligning with the observations of Casper and Stimper (2009) and correcting earlier misreports by Zamora *et al.* (1996), who listed $2n=48$ for these taxa. Similarly, we have validated the chromosome counts for *P. casperiana* and *P. christinae*, confirming their octoploid status ($2n=64$), as previously reported by Casper and Stimper (2009). Although *P. christinae* is also reported as hexadecaploid ($2n=128$, Casper and Stimper 2006: table 1 ‘*P. cf. vulgaris*: Mt. Corchia; Foce della Verginetta’), we could not confirm this, but do not rule it out. It would indicate spontaneous polyploidization within a hybrid taxon (see below). Our results are corroborated by genome size (AGS) measurements and molecular analyses, which place these taxa within the octoploid cluster. Genome size variation among octoploid taxa shows a 1.5-fold range, from *P. ernica* ($2C=1.32$ pg) to *P. vallis-regiae* ($2C=1.97$ pg). However, no clear pattern links genome size to evolutionary relationships within octoploids.

For tetraploid taxa, we confirm *P. nevadensis* as tetraploid ($2n=32$), consistent with Casper and Stimper (2009) but contradicting Zamora *et al.* (1996), who reported $2n=16$. Despite having the smallest genome among the investigated taxa, the genome size of *P. nevadensis* is not sufficiently distinct to suggest

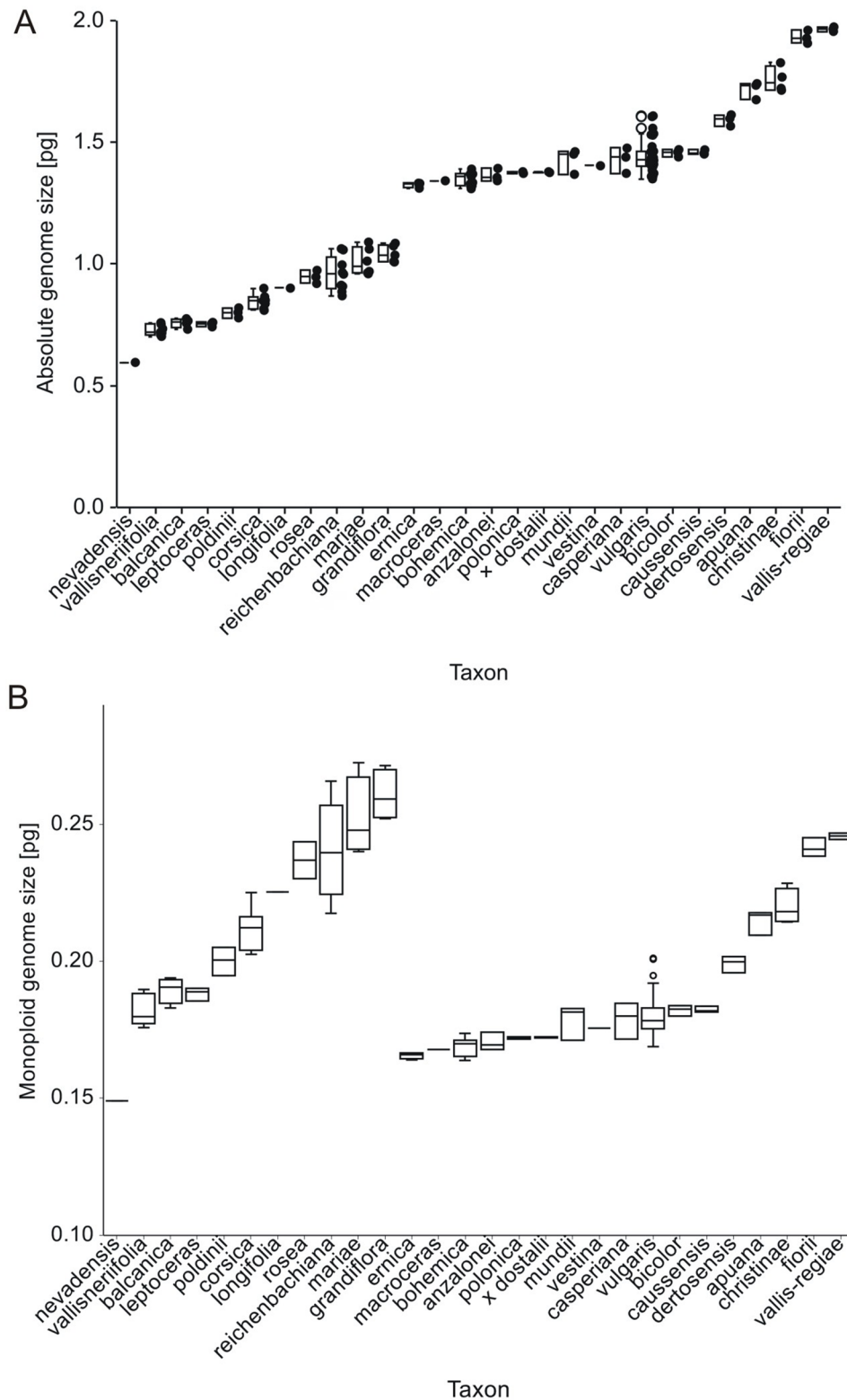


Figure 5. The variation of genome size in the studied taxa. Species are sorted according to their mean genome size. A, absolute genome size; B, monoploid genome size.

a diploid ploidy level. Another taxon that requires clarification regarding correct chromosome reporting is *P. longifolia*. This species is tetraploid, and the first report of chromosome count is often referred to as Doulat (1947) (e.g. see Casper and Stimper 2009:

table 1). However, this is not correct. Doulat counted chromosomes in a specimen of *P. reichenbachiana* [reported in the paper as '*P. longifolia* Ram. var. *Reichenbachiana* (Schindl.) Rouy'] from the locality 'Alpes-Maritimes, vallée de la Roja, entre de Tende et

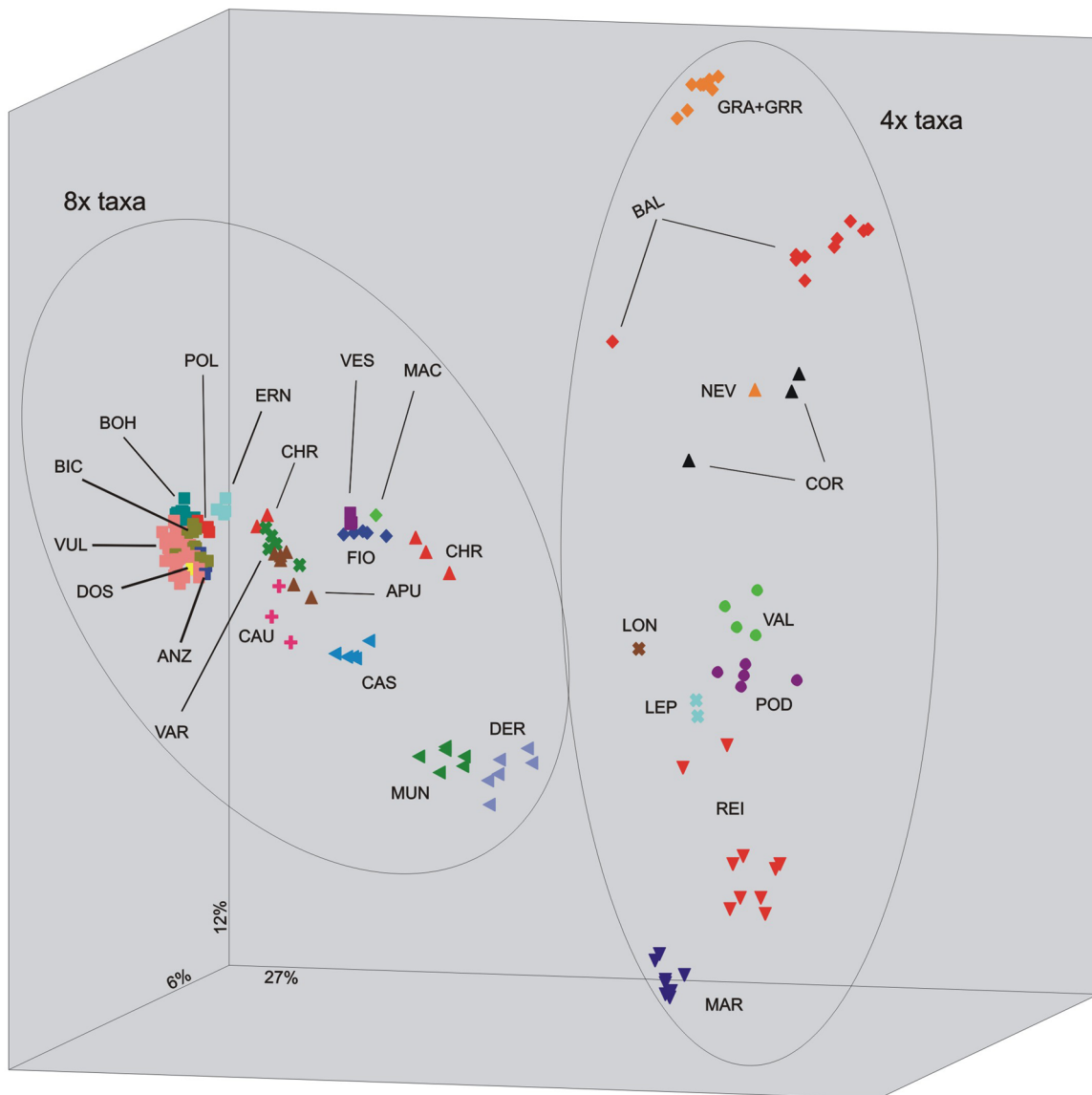


Figure 6. Principal coordinate analysis (PCoA 3D plot, based on Jaccard's similarity coefficient). The first three axes explain 45% of the total variation.

Fontan, 450 m' (Doulat 1947: 355). It is evident that it is outside the range of *P. longifolia*, which is endemic to the province of Huesca in the Spanish Pyrenees (Table 1). Consequently, the first accurate count for *P. longifolia* should be attributed to Zamora *et al.* (1996).

The most surprising result is the tetraploid chromosome count for *P. corsica*. To our knowledge, there are only two original reports on the chromosome number for *P. corsica*, both from the studies of Contandriopoulos (1957, 1962), both of which show $2n = 16$ chromosomes. Based on these studies, this species is considered to be diploid ($2n = 16$), which was accepted and further disseminated mainly through the 'Monographie der Gattung *Pinguicula* L' (Casper 1966). All other commonly used references, e.g. Casper (1962a, 1963), Bolkhovskikh *et al.* (1969), Yoshimura (1973), Moore (1982), and Steiger (1998), are either wrong or do not provide original counts, but represent a secondary or tertiary citation referring to the above-mentioned works by Contandriopoulos. However, the

most recent and comprehensive karyological study of the genus *Pinguicula* should have provided a new original report on the number of chromosomes for *P. corsica*, supporting the diploidy of this species (Casper and Stimper 2009: table 1), but no further clarification of this claim was provided, e.g. the image documentation. Although our analysis of samples from several localities consistently suggests that *P. corsica* is tetraploid, we cannot exclude the possibility of cytotype variation. The $2n = 16$ reported for *P. schuensis* is also questionable, as the evidence is not convincing (Bacchetta *et al.* 2014: fig. 3), and this taxon requires further analysis.

This study also presents the first genome size estimates for nine taxa (Table 2). Previously, genome sizes had only been reported by Veleba *et al.* (2014) and Majesky *et al.* (2022). A comparison of the genome sizes with the values from Veleba *et al.* (2014) revealed discrepancies, which can be attributed to differences in internal standards (cf. Šmarda *et al.* 2019). However, after recalculating the data from Veleba *et al.* (2014) using the internal

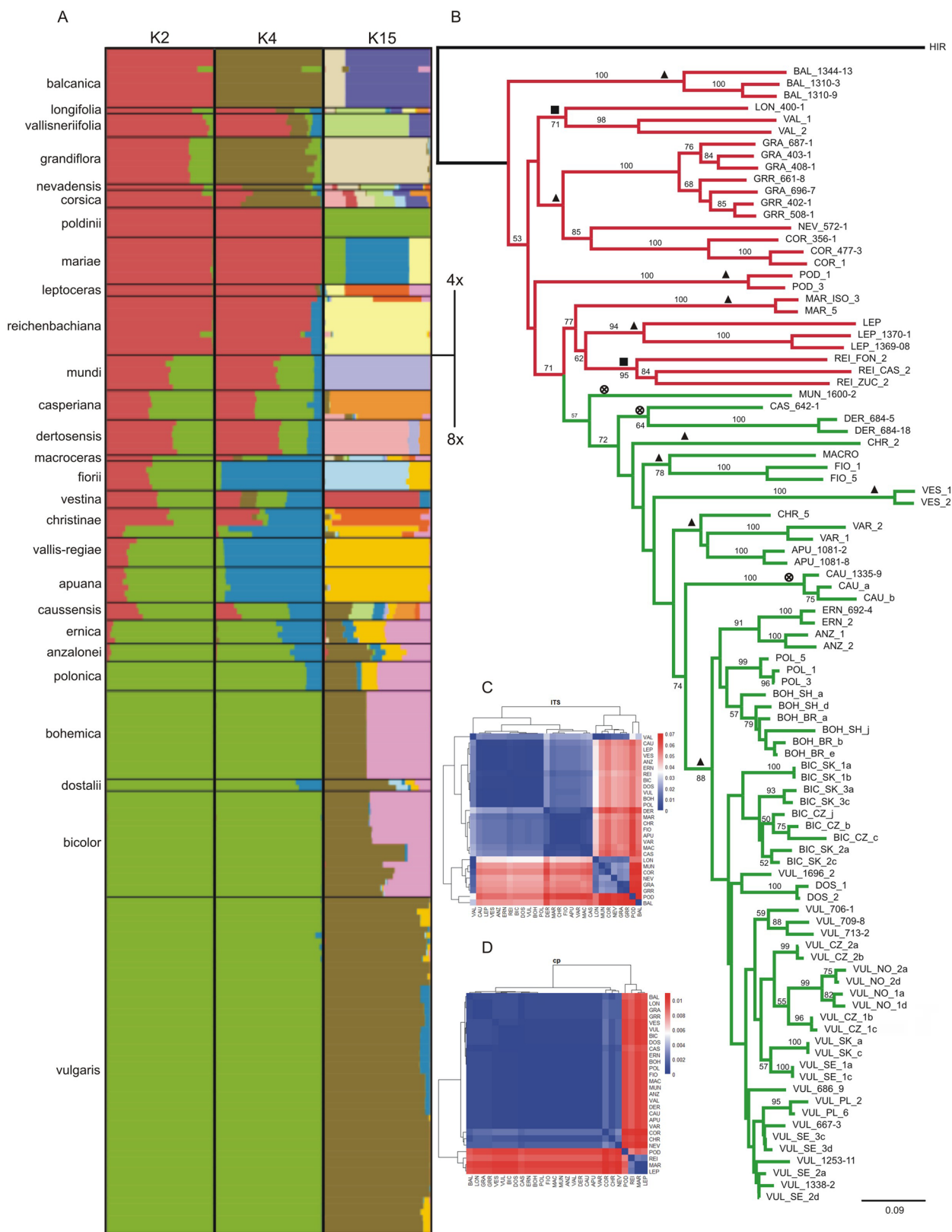


Figure 7. Results of clustering analyses. A, structure clustering for $K=2$, $K=4$, and $K=15$. Each individual is represented by a vertical bar, the colour representing the probability of assignment to different clusters, and each clustering is based on 10 replicate runs. B, dendrogram of neighbour-joining analysis of merged AFLP (520 markers) and ITS (195 markers) data (Jaccard coefficient of similarity) rooted by *P. hirtiflora*. Bootstrap support >50 is shown above/below branches. Coloured branches denote the ploidy level: red—tetraploid ($2n=32$), green—octoploid ($2n=64$). The symbols above the branches indicate the rosette type: square—anisophyllous, triangle—isophyllous, circle with cross—slightly anisophyllous. C, heatmap showing clustering according to genetic distances (p-distances) derived from the sequences of the ITS locus. D, heatmap showing clustering according to genetic distances (p-distances) derived from the sequenced cpDNA loci.

standards applied in this study (following Doležel *et al.* 2007), the differences were minimized to just 0.00–0.03 pg (Table S6).

The weak observed correlation of the monoploid genome size suggests that other factors influence the relationship between genome size and latitude and longitude. The smaller monoploid genome size observed in advanced octoploid taxa with a wider northern distribution may be due to selective pressure acting upon them

Table 3. Sequence polymorphism observed within the investigated regions.

Locus	N	Length	CON	VAR	PI	NucDiv
<i>trnT-trnF</i>	110	901	885	8	8	0.0020
8x	81	820	818	2	2	0.0008
4x	29	901	886	7	7	0.0030
<i>petN-psbM</i>	114	1852	1787	33	33	0.0020
8x	86	1839	1804	3	3	0.0003
4x	28	1852	1789	31	31	0.0060
<i>trnQ-rps16</i>	119	682	656	19	18	0.0020
8x	91	614	613	1	1	0.0001
4x	28	674	649	19	18	0.0080
<i>trnK-matK</i>	111	1082	1067	15	15	0.0010
8x	83	1082	1079	3	2	0.0001
4x	28	1082	1068	14	14	0.0040
<i>ITS1–5.8S–ITS2</i>	116	624	514	107	90	0.0190
8x	86	619	572	45	16	0.0060
4x	28	624	524	97	83	0.0400
<i>Altogether</i>	n.a.	5141	4909	182	164	n.a.

CON/VAR/PI, number of conserved/variable/parsimony-informative sites; length, length of the final alignment in number of nucleotides; N, number of individual sequences; n.a., not analysed; NucDiv, nucleotide diversity.

following the polyploidization event, as opposed to octoploid taxa from lower latitudes (Wang *et al.* 2021, Bureš *et al.* 2024). However, it may also reflect their hybrid origin from tetraploids with smaller 1Cx (involving e.g. *P. leptoceras*). The larger 1Cx in some tetraploid taxa may be due to their longer isolation, restricted gene flow, smaller effective population size, and subsequent proliferation of retroelements, a scenario under the mutational hazard hypothesis (Lynch and Conery 2003, Bureš *et al.* 2024). Conversely, different 1Cx values in closely related tetraploid taxa may be linked to different life histories of diploid parental taxa and reflect different rates of postpolyploidization changes (Mandáková *et al.* 2017). While all these hypotheses may contribute to the observed intricate pattern, we currently have insufficient evidence to support any of them.

Phylogenetic relationships and biogeography of *P. sect. Pinguicula*

The use of molecular markers in butterwort biosystematics has changed the view of the traditional classification introduced by Casper (e.g. Casper 1962a, 1963, 1966), who emphasized the importance of flower morphology. Recent studies on the pollination biology of butterworts have shown that flower morphology, the characters enforced by Casper, is tuned to the main group of pollinators (Lustofin *et al.* 2020). This suggests that similarities in floral traits may not reflect evolutionary relationships, but rather instances of parallel evolution (e.g. Cieslak *et al.* 2005, Degtjareva *et al.* 2006, Fleischmann 2021, Shimai *et al.* 2021). This is even supported by our data, indicating the repeated and independent occurrence of anisophylly within otherwise isophyllous species of *P. sect. Pinguicula*. An isophyllous rosette probably represents the plesiomorphic state for this group (Fig. 7B; Fig. S2).

Table 4. Patterns of duplication sharing among the investigated butterwort species.

Locus	Duplicated motif	1× DUP	2× DUP	4× DUP
<i>psbM-trnC</i>	16 bp [TATTCTAATCTACTCG]	NEV	ANZ, APU, BIC, BOH, CAS, CAU, CHR, DER, DOS, ERN, FIO, GRA , GRR , LEP , LON , MUN, POL, VAL , VAR, VES, VUL	MAC
	6 bp [CTAATG]	COR	×	×
	4 bp [AAAT]	ANZ, APU, BIC, BOH, CAS, CAU, CHR, COR , DER, DOS, ERN, FIO, GRA , GRR , LON , MAC, MUN, NEV , POL, VAL* , VAR, VES, VUL	×	×
<i>trnQ-rps16</i>	7 bp [TAGTATG]	BOH, POL	×	×
	23 bp [TGTCCAATTATTCAAATTAA]	LEP_1 , REI_FON	×	×
	8 bp [AAATTAAA]	POD	×	×
	6 bp [AATTTC]	POD , MAR , REI , LEP	×	×
	15 bp [AATTATATAATTCTA]	MAR , REI	LEP	×
<i>trnT-trnF</i>	7 bp [AAATATA]	LEP , MAR	×	×
	14 bp [AATATATATTGAAT]	LEP , MAR , REI	×	×
	8 bp [ATATATAC]	LEP , MAR , POD , REI	×	×
	32 bp [AGTCAATCTTAAATACCTTTAAGATAGTCAAA]	COR	×	×
	8 bp [TTAACGAA]	LEP , REI , MAR	×	×
	8 bp [TCTAATAA]	VAL_2	×	×

Tetraploid taxa are highlighted in bold; for taxon/population abbreviations see Appendix 1.

bp, duplication length in the number of nucleotide pairs; 1×/2×/4× DUP, number indicating how many copies were observed for a given taxon; ×, not present.

Fleischmann and Roccia (2018) correctly pointed out that Casper's classification has produced groups of paraphyletic and polyphyletic origin. The combination of nuclear ITS region and chloroplast regions (Cieslak et al. 2005, Degtjareva et al. 2006, Shimai et al. 2021) has resolved the major clades within the genus that correspond to subgenera and sections (Fleischmann and Roccia 2018), but relationships within the clades remain largely unresolved (e.g. De Castro et al. 2016, Crespo et al. 2019, Shimai et al. 2021). The topology of the inferred trees in these studies remains consistent but shows polytomies at the lower taxonomic level. These discrepancies are probably due to low genetic differentiation, especially among octoploid taxa (see below). The studied *P. sect. Pinguicula* is thought to be a relatively recent group within the genus, and its origin and diversification are closely linked to Pleistocene climatic oscillations (e.g. Casper 1966, Casper and Stimper 2009). The evolutionary dynamics that have shaped the current patterns observed in the genus are driven primarily by hybridization, polyploidization and geographical isolation (Fleischmann 2021, Shimai et al. 2021, Majesky et al. 2022).

The origin of tetraploid taxa

Our analyses clearly distinguish between the two cytotypes within *P. sect. Pinguicula*: tetraploid ($4x$) and octoploid ($8x$), with tetraploids representing the basal group and octoploids being derived. Within each cytotype, distinct groups can be identified. The most recent common ancestor of *P. sect. Pinguicula* probably consisted of a few diploid taxa, now extinct or restricted to remote Mediterranean mountainous areas and yet to be recognized (Fig. 8). Considering the distribution of the extant taxa, the Mediterranean region, especially the Apennine and Iberian peninsulas, could be considered as the evolutionary cradle of *P. sect. Pinguicula*. Here, the group probably started to diversify at the end of the Tertiary (Ibarra-Laclette et al. 2013, Silva et al. 2018), and the process continued throughout the Pleistocene.

Tetraploids may have originated by chromosome duplication within hybrid swarms of interbreeding diploid ancestors. Similarly, the octoploid cytotype probably arose via duplication within tetraploid hybrid swarms. The possibility of autopolyploidy in these cytotypes also cannot be ruled out (Fig. 8). This polyploidization stabilized early hybridization products, allowing them to overcome sterility or some other genomic instability. As noted by Fleischmann (2021), there are no significant barriers, other than geographical separation, preventing hybridization among taxa within *Pinguicula* subgenera or sections. Where species occur sympatrically, hybridization is common (see 'natural hybrids' in Table 1). However, hybridization between $4x$ and $8x$ cytotypes contributes minimally to species diversity within *P. sect. Pinguicula*. Such crosses would produce hexaploid hybrids (Fig. 8), which may have meiotic problems due to irregular chromosome pairing (Roccia 2013). A good example is *P. × scullyi* nothosubsp. *gresivaudanica* (Roccia) Schlauer, a hybrid between tetraploid *P. grandiflora* subsp. *rosea* and octoploid *P. vulgaris* (Roccia 2013, Schlauer 2013), which is sterile and spreads only vegetatively (Roccia 2013). This probably explains the absence of well-established hexaploid taxa within the section. The hexaploidy reported by Zamora et al. (1996) for *P. mundi* and *P. submediterranea* Blanca, Jamilena, Ruíz Rejón & Reg. Zamora was later shown to be erroneous (Casper and Stimper 2009, the present study), although it cannot be excluded that the analysed individuals represent hybrids between some of the $8x$ and $4x$ taxa.

The observed clustering pattern, sequence polymorphism, and shared duplications suggest the presence of distinct genetic lineages between the tetraploid taxa. This suggests that independent evolutionary events have repeatedly produced the tetraploid cytotype. However, the available data do not provide a clear timeline for these occurrences. One identified cluster groups together the Iberian tetraploid taxa *Pinguicula nevadensis*, *P. longifolia*, and *P. vallisneriifolia* along with *P. corsica*. The inferred topologies of the AFLP + ITS and AFLP trees show slight differences, as they additionally place *P. grandiflora* or *P. balcanica* within this cluster (Fig. 7B; Fig. S2). While *P. vallisneriifolia* and *P. longifolia* can be considered evolutionarily closely related, *P. corsica*, *P. nevadensis*, *P. grandiflora*, and *P. balcanica* represent more distinct genetic lineages. The shared clustering of these species suggests they originated from a common genetic pool, at least in part (Fig. 8). *Pinguicula balcanica* appears to be an orphan lineage that became isolated early after their divergence, and has become a geographically vicariant species, replacing the tetraploid taxa in the Balkan Mountains. Another distinct cluster consists of the remaining tetraploid taxa *P. mariae*, *P. reichenbachiana*, and *P. leptoceras*, which are clearly closely related. Although neighbour-joining analysis of AFLP data did not directly place *P. poldinii* within this cluster (Fig. S2), the combined AFLP+ITS data suggest a close relationship with these three species (Fig. 7B). *Pinguicula poldinii* probably represents a basal or earlier diverging and isolated taxon within this tetraploid group (Fig. 8). Similar groupings of these taxa have been reported in previous studies (e.g. Crespo et al. 2019, 2020).

The origin of octoploid taxa

The low sequence divergence among octoploid taxa suggests their origin from a very restricted gene pool, comprising only a few genetically similar taxa. Together, the observed pattern of clustering, sequence polymorphism, and duplication sharing suggests the origin of the octoploid cytotype as a single event with gradual diversification of the extant taxa. However, an alternative scenario, also supported by the data, suggests that the $8x$ cytotype arose repeatedly. There are two distinct groups of octoploid taxa, which differ mainly in their geographical distribution and the parental taxa involved in their origin (Fig. 8). The Iberian taxa *P. mundi*, *P. dertosensis*, and *P. casperiana* represent one group, in which *P. mundi* is the basal species. *Pinguicula mundi* could have arisen either by hybridization between $4x$ taxa genetically close to *P. nevadensis*, *P. longifolia*, and *P. vallisneriifolia* and subsequent polyploidization, or by autopolyploidization of some of these tetraploids (Fig. 8). This inference is based on the similarity of ribotypes and haplotypes of *P. mundi* to these $4x$ taxa (Fig. 7D, C; Table S5). The remaining two taxa (*P. casperiana* and *P. dertosensis*) subsequently arose from crosses between *P. mundi* and some of the $8x$ taxa that migrated westwards to the Iberian Peninsula (Fig. 8). The most likely candidate is widely distributed *P. vulgaris*, which has been reported from the Pyrenees (Blanca et al. 1999). Therefore, it would be interesting to sequence Iberian samples of *P. vulgaris* to see if they match the observed typical genotype of *P. vulgaris* agg. or represent another taxon. Due to the lack of available material, we did not include other Iberian taxa (*P. saetabensis* M.B. Crespo, Mart.-Azorín & M.Á. Alonso, *P. submediterranea* Blanca, Jamilena, Ruiz-Rejón & R. Zamora, *P. tejedensis* M.B. Crespo, M. Azorín, M.Á. Alonso & L. Sáez) in our analyses, so the

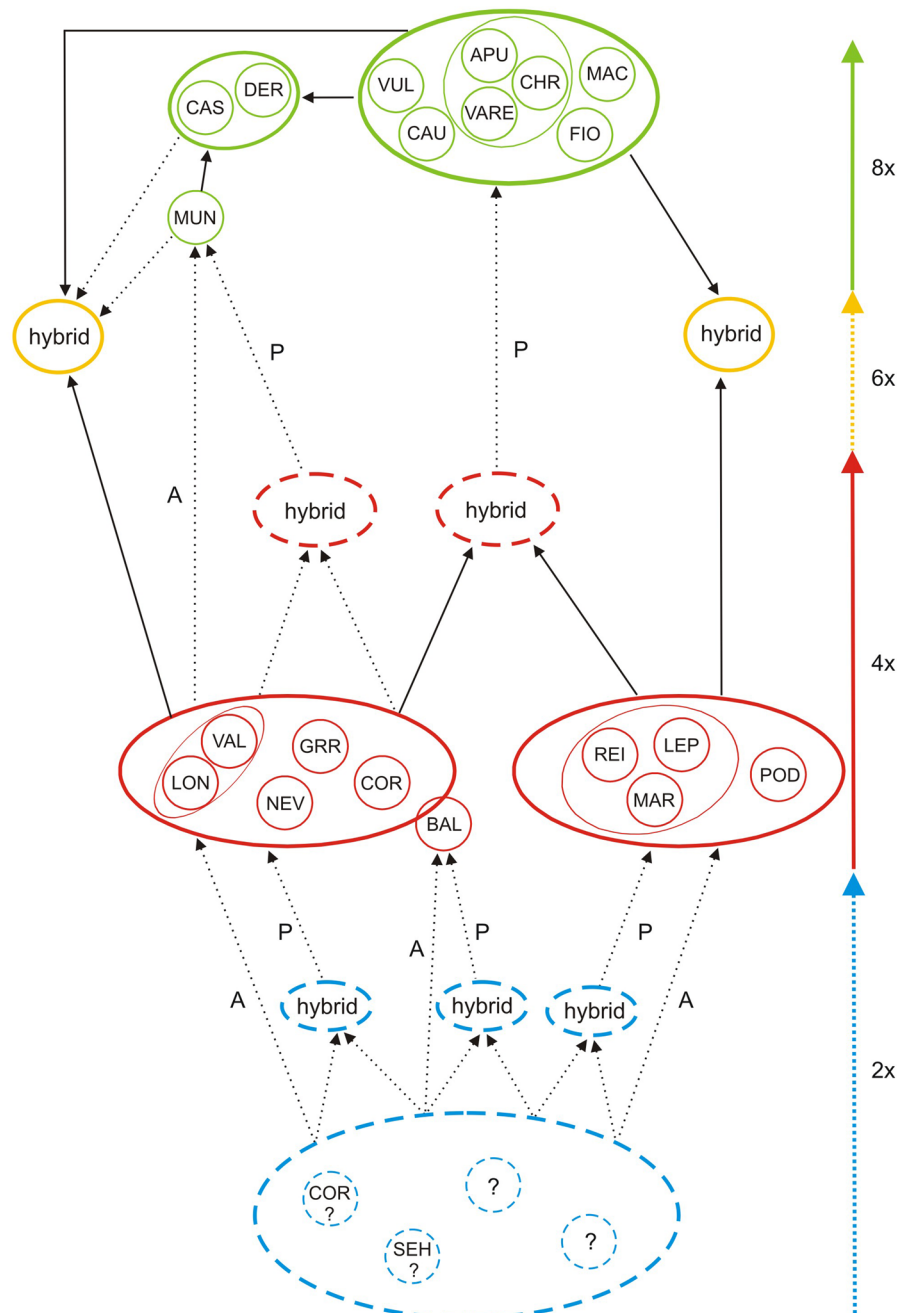


Figure 8. The proposed scenario of the diversification process within *P. sect. Pinguicula* with the gradient of ploidy levels (on the right margin). Dashed ovals/dotted lines represent hypothetical taxa/processes, while solid lines represent recent taxa/processes supported by the presented data. Taxa are grouped based on proposed relationships. In the case of the formation of hexaploid hybrid taxa, although such hybrid populations are known (see text), they have not been karyologically studied, so this ploidy is indicated as hypothetical on the right margin. The diploid level is indicated as hypothetical because our results support tetraploidy but not diploidy for *P. corsica*, which was previously reported as diploid. Diploidy in *P. sehuensis* requires further study (see the text). A, autopolyploidization; P, polyploidization in a hybrid swarm.

presented relationships within these Iberian taxa may be slightly different.

The remaining octoploid taxa form a second group that probably originated in a similar manner but from a different parental tetraploid gene pool. Most of these taxa have ribotypes similar to *P. leptoceras*, *P. mariae*, and *P. reichenbachiana* but haplotypes similar to the 4x Iberian taxa (Fig. 7C, D; Table S5). Based on maternal inheritance of cpDNA, a plausible ancestral

combination involves a 4x taxon genetically similar to *P. grandiflora*, *P. longifolia*, or *P. vallisneriifolia* as the seed parent, and a 4x taxon genetically similar to *P. leptoceras*, *P. mariae*, or *P. reichenbachiana* as the pollen donor. Polyploidization within the resulting hybrid swarm probably gave rise to the basal 8x taxon/taxa (Fig. 8). Subsequent diversification at the octoploid level was probably driven by geographical isolation during Quaternary climatic oscillations. These events reconnected and then

separated previously isolated lineages, facilitating hybridization between taxa at the same ploidy level and the emergence of new lineages. The relationships among the Apennine taxa *P. apuana*, *P. christinae*, and *P. vallis-regiae* exemplify such speciation. In our analyses, these three taxa cluster together, although *P. christinae* appears in two separate positions on both the AFLP and AFLP + ITS trees (Fig. 7B; Fig. S2), despite the samples originating from the same population. This suggests that *P. christinae* has a recent hybrid origin and has not yet been stabilized by concerted evolution, a hypothesis previously proposed also by its discoverers, Peruzzi and Gestri (2013), and later by Roccia et al. (2016). While our data do not definitively identify the parents of *P. christinae*, likely candidates are *P. apuana* and *P. vallis-regiae*. If the reported $2n = 128$ chromosome number for *P. christinae* is correct (Casper and Stimper 2009), it would represent spontaneous polyploidization within a hybrid taxon, supporting the evolutionary pathway described above and the role of polyploidization in butterwort diversification.

Pinguicula macroceras and *P. vulgaris*

Pinguicula macroceras and *P. vulgaris* are the only two taxa of the *P.* subgenus *Pinguicula* growing in North America, and *P. macroceras* has long been considered to be conspecific with *P. vulgaris* or a geographical vicariant of *P. vulgaris* in the Amphi-Pacific region, where *P. vulgaris* is absent (Casper 1962b, 1966). However, our analyses clearly show that *P. macroceras* is distinct from *P. vulgaris*, with both taxa having independent evolutionary origins (Fig. 7B; Fig. S2). The native range of *P. macroceras* includes Japan across the Kamchatka Peninsula to Alaska, Oregon, and California (Casper 1962b, 1966). In contrast, in North America, *P. vulgaris* is distributed across Labrador and much of Canada, and extends westward to Alaska (Casper 1966, Roccia et al. 2016). Alaska probably acts as a contact zone for these two taxa, but their precise ranges remain uncertain due to historical and ongoing taxonomic confusion (Casper 1962b, 1966).

Our AFLP analysis indicates a close relationship between *P. fiorii* and *P. macroceras* (Fig. 7B; Fig. S2), whereas ITS sequence data suggest affinities with *P. casperiana* and *P. dertosensis* (Fig. S3). Nevertheless, the quadruple duplication of the 16-bp minisatellite within the *petN-psbM* locus distinguishes *P. macroceras* as a unique taxon (Table 4). Morphologically, *P. fiorii* and *P. macroceras* are similar in flower size and shape (Casper 1962a, Peruzzi and Gestri 2013), yet their very different geographical distributions are not readily explained. Notably, these species represent extremes of estimated genome size among the investigated octoploid taxa (Table 2): *P. macroceras* possesses the second smallest genome size ($2C = 1.34$ pg), whereas *P. fiorii* has the second largest ($2C = 1.93$ pg).

It appears that *P. macroceras* represents an isolated lineage that originated from the ancestral gene pool of all subsequent octoploid taxa. This lineage may have expanded eastward, establishing itself in Northeast Asia before dispersing across Beringia into North America. In contrast, *P. vulgaris* probably colonized North America during the Pleistocene via North Atlantic islands serving as stepping stones from Europe. Resolving the taxonomic and evolutionary status of *P. macroceras* will require comprehensive sampling across its range.

The origin of *P. vulgaris* also remains uncertain, but the identification of *P. caussensis* as a sister species to the broader *P. vulgaris* group (Fig. 7; Fig. S2) suggests that the genetic lineage now recognized as *P. caussensis* may have played a role in the origin of *P. vulgaris*. This hypothesis is supported by the high similarity of ITS sequences, differing by only a single transition (272: C→T) from the ribotype typical of all *P. vulgaris* members studied (Table S5). *Pinguicula vulgaris* agg. constitutes a complex of closely related taxa that evolved through hybridization and geographical isolation (Majeský et al. 2022). These taxa are considered among the most evolutionarily recent members of *P.* sect. *Pinguicula*, originating during the Pleistocene (Casper 1966, Casper and Stimper 2009). Of particular interest is the position of *P. vulgaris* subsp. *vestina* in the inferred trees. Although it has the ITS ribotype characteristic of the *P. vulgaris* complex (Table S5), several analyses highlight its distinctiveness (e.g. Figs 6, 7; Fig. S2). This taxon is probably of hybrid origin, resulting from a hybridization within *P.* sect. *Pinguicula* ($8x$).

CONCLUSION

Our study provides a comprehensive overview of chromosome numbers and genome size estimates for the majority of taxa within *P.* sect. *Pinguicula*. Consistent with the literature, our findings indicate the presence of two dominant ploidy levels, tetraploid ($2n = 32$) and octoploid ($2n = 64$). Most previous reports of other ploidy levels are probably erroneous, given the challenges associated with chromosome counting in *Pinguicula*. However, some taxa (e.g. *P. corsica*, *P. sehuensis*) and regions (e.g. Mediterranean islands) for which $2n = 16$ have been reported require further investigation. From an evolutionary perspective, our results highlight the limitations of traditional phylogenetic inference based on a few genetic markers. A more comprehensive approach utilizing modern whole-genome techniques is essential for deeper insights into butterwort phylogeny. Our analyses clearly distinguish two cytotypes within *P.* sect. *Pinguicula*, with tetraploid taxa forming the basal group and octoploids representing derived lineages. Our results strongly support the presence of different tetraploid lineages that gave rise to the advanced octoploid taxa. However, the ancestral gene pool from which all extant octoploid taxa arose was probably highly restricted. While we propose potential evolutionary relationships (Fig. 8), these should still be interpreted with caution due to the low molecular, morphological, and ecological differentiation among the studied taxa and the relatively limited sample size. Future research integrating broader sampling will be crucial in fully resolving these evolutionary relationships.

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SUPPLEMENTARY DATA

Supplementary data are available at Botanical Journal of the Linnean Society online.

CONFLICT OF INTEREST

None declared.

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DATA AVAILABILITY

The nucleotide sequences were submitted to GenBank (see Table S3). The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Appendix 1. Sampling details of the studied taxa of *Pinguicula* sect. *Pinguicula*.

Taxon/code	Country	Locality	N (AFLP)	N (cp/ITS)	N (GS)
<i>P. apuana</i> Casper & Ansaldi (APU)					
APU_1081	IT	Alpi Apuane, Mt Corchia	6	2	3
<i>P. balcanica</i> Casper (BAL)					
BAL_1310	BG	Pirin Mts, Mt Vihren	6	2	2
BAL_1344	GR	Mt Voras	4	1	2
<i>P. casperiana</i> M.B. Crespo, Mart.-Azorín, M.Á. Alonso & L. Sáez (CAS)					
CAS_642	ES	Hoz de Beteta, Sierra de Cuenca	1	1	1
CAS_1373	ES	Priego, Sierra de Cuenca	4	1	2
<i>P. caussensis</i> (Casper) Roccia, Gluch, Lampard, A.S. Rob., A. Fleischm., S. McPherson, L. Legendre, Partrat & P. Temple (CAU)					
CAU_1335	FR	Gorges du Tarn	1	1	1
CAU_a/b	FR	Gorges du Tarn	2	2	2
<i>P. christinae</i> Peruzzi & Gestri (CHR)					
CHR	IT	Tuscany, Lama Rossa	5	2	4
<i>P. corsica</i> Bernard & Gren. ex Gren. & Godr. (COR)					
COR_356	FR	Lavu di l'anguille	1	1	0
COR_477	FR	Mt Cinto	1	1	1
COR_1	FR	Mt Rotondo	1	1	6
<i>P. dertosensis</i> (Cañig.) Mateo & M.B. Crespo (DER)					
DER_684	ES	Tortosa, near Cua de Cavall	6	2	3
<i>P. fiorii</i> Tammara & L. Pace (FIO)					
FIO	IT	Maiella Mts, Mt Focalone	5	2	3
<i>P. grandiflora</i> LAM. subsp. <i>grandiflora</i> (GRA)					
GRA_408	FR	Pyrenees, Aragnouet, near village Fabian	1	1	1
GRA_403	FR	Pyrenees, near road D118	1	1	1
GRA_696	FR	Department Isère, near village Rovon	1	1	1
GRA_689	AD	Pyrenees, Coma Pedrosa	0	0	1
GRA_687	ES	Pyrenees, Ordesa, Circo de Goriz	1	1	1
<i>P. grandiflora</i> subsp. <i>rosea</i> (Mutel) Casper (GRR)					
GRR_661	FR	between Grenoble and Chambéry	3	3	1
GRR_402	FR	between Grenoble and Chambéry	1	1	1
GRR_509	FR	between Grenoble and Chambéry	1	1	0
GRR_508	FR	between Grenoble and Chambéry	1	1	1
<i>P. leptoceras</i> Rchb. (LEP)					
LEP	FR	Alpes Maritimes, La Monta	1	1	0
LEP_1370	CH	near San Bernardino	1	1	2
LEP_1369	IT	Valstrona	1	1	1
<i>P. longifolia</i> Ramond ex. DC. (LON)					
LON	FR	Pyrenees, Gavarnie	1	1	1
<i>P. macroceras</i> Link (MAC)					
MAC	JP	Akita, Mt Nyuto (Mt Eboshi)	1	1	1
<i>P. mariae</i> Casper (MAR)					
MAR	IT	between Castelnuovo di Garfagnana—Massa	5	1	3
ISO	IT	Isola Santa	3	1	3

(Continued)

Appendix 1. (Continued)

Taxon/code	Country	Locality	N (AFLP)	N (cp/ITS)	N (GS)
<i>P. mundi</i> Blanca, Jamilena, Ruíz Rejón & Reg. Zamora (MUN)					
MUN_1600	ES	Rio Mundo, Cueva de los Chorros	6	2	3
<i>P. poldinii</i> J. Steiger & Casper (POL)					
POD	IT	Tolmezzo, near village San Francesco	5	2	3
<i>P. nevadensis</i> (H. Lindb.) Casper (NEV)					
NEV_572	IT	Sierra Nevada, near village Trevélez	1	1	1
<i>P. reichenbachiana</i> J. Schindl. (REI)					
CAS	IT	Castelvechio di Rocca Barbena, Colle Scravaion	3	2	3
ZUC	IT	near town Zuccarello	3	2	3
FON	FR	Roya valley, Fontan, loccus classicus	3	2	3
<i>P. vallisnerifolia</i> Webb (VAL)					
VAL_647	ES	Sierras de Segura, near Laguna de Aguas Negras	2	1	1
VAL_1	ES	Sierras de Segura, near Vadillo Castril	1	1	3
VAL_2	ES	Sierras de Segura, near Vadillo Castril	1	1	3
<i>P. vallis-regiae</i> F. Conti & Peruzzi (VAR)					
VAR	IT	Camosciara	5	2	3
<i>P. vulgaris</i> L. subsp. <i>vulgaris</i> (VUL)					
VUL_SE_1	SE	Along the lake Kyrksjön	3	2	0
VUL_SE_2	SE	Near the town Högländ	4	2	1
VUL_SE_3	SE	Near the town Fagersta	4	2	0
VUL_SE_4	SE	Along the road N°344 and lake Otsjön	0	0	1
VUL_NO_1	NO	In between towns Haugastøl and Ustaostat	4	2	0
VUL_NO_2	NO	Along the lake Varaldsjøen	4	2	0
VUL_CZ_1	CZ	Šumava Mts, near Nové Údolí town	3	2	0
VUL_CZ_2	CZ	Šumava Mts, near Keply town	4	2	3
VUL_SK	SK	Near Vážec village	4	2	0
VUL_SK_4	SK	Veľká Fatra Mts, Blatnica, Dedošova dolina	0	0	2
VUL_SK_5	SK	Lubovnianska vrchovina Mts, near Litmanova village	0	0	2
VUL_1253	SK	Veľká Fatra Mts, near the town Ružomberok	1	1	1
VUL_MP	SK	Muránska planina, near village Telgárt	0	2	0
VUL_1696	DE	Bavaria, near Wallgau village	6	1	3
VUL_GAP	DE	Bavaria, near Garmisch-Partenkirchen	0	4	4
VUL_PL	PL	Zakopane	6	2	3
VUL_TUR	PL	Gorce Mts, Turbacz	0	4	0
VUL_686	UK	Wales, Snowdonia	3	1	1
VUL_1338	IE	Downings, Co. Donegal	5	1	3
VUL_CH	CH	Canton of Vaud, near the town Vevey	2	1	2
VUL_A	CH	Conthey, mountains above Daillon	0	1	2
VUL_706	FR	Cottian Alps, Col de l'Agnelin	1	1	1
VUL_709	FR	Isère, near lake 'Lac Fourchu'	1	1	1
VUL_713	FR	Isère, Theyes, peat bog near the town	1	1	1
VUL_A	FR	L'Argentière-la-Bessée, around the path to Crête de la Baume Noire	0	2	1
VUL_E	FR	Saint-Paul-sur-Ubaye, Route du Vallon de Maurin	0	1	1
VUL_IS	IS	Skagi peninsula, around the lake Selvatn	0	3	0
<i>P. vulgaris</i> var. <i>bicolor</i> Nordst. ex Fr. (BIC)					
BIC_SK_1	SK	Malá Fatra Mts, near Vrícko village	3	2	0
BIC_SK_2	SK	Malá Fatra Mts, near village Stankovany	3	2	0
BIC_SK_3	SK	Chočské vrchy Mts, near village Valaská Dubová	3	2	0
BIC_SK_6	SK	Malá Fatra Mts, Klačno	0	0	1
BIC_CZ	CZ	Štramberk town, stone-pit 'Dolní Kamenárka'	10	4	2
<i>P. vulgaris</i> subsp. <i>bohemia</i> (Kraj.) Domin (BOH)					
BOH_SH	CZ	NW Bohemia, Shnilé louky	10	2	9
BOH_BR	CZ	NW Bohemia, Baronský rybník	5	2	2
<i>P. vulgaris</i> nothosubsp. <i>dostalii</i> (Bárta) ined. (DOS)					
DOS	CZ	Mělnická Vrutice, Polabská Černava	2	2	2
<i>P. vulgaris</i> 'polonica' ined. (POL)					
POL	PL	Woj. śląskie, Dąbrowa Górnicza, abandoned sandpit	5	3	2

(Continued)

- Fu CN, Wicke S, Zhu AD *et al.* Distinctive plastome evolution in carnivorous angiosperms. *BMC Plant Biology* 2023;**23**:660. <https://doi.org/10.1186/s12870-023-04682-1>
- Fukushima K, Fang X, Alvarez-Ponce D *et al.* Genome of the pitcher plant *Cephalotus* reveals genetic changes associated with carnivory. *Nature Ecology & Evolution* 2017;**1**:59. <https://doi.org/10.1038/s41559-016-0059>
- Greilhuber J, Doležel J, Lysak MA *et al.* The origin, evolution and proposed stabilization of the terms 'genome size' and 'C-value' to describe nuclear DNA contents. *Annals of Botany* 2005;**95**:255–60. <https://doi.org/10.1093/aob/mci019>
- Hedrich R, Fukushima K. On the origin of carnivory: Molecular physiology and evolution of plants on an animal diet. *Annual Review of Plant Biology* 2021;**72**:133–53. <https://doi.org/10.1146/annurev-arplant-080620-010429>
- Hintze J. NCSS 9. Kaysville, Utah, USA: NCSS, LLC, 2013.
- Ibarra-Laclette E, Lyons E, Hernández-Guzmán G *et al.* Architecture and evolution of a minute plant genome. *Nature* 2013;**498**:94–8. <https://doi.org/10.1038/nature12132>
- Jakobsson M, Rosenberg NA. CLUMPP: a cluster matching and permutation program for dealing with label switching and multimodality in analysis of population structure. *Bioinformatics* 2007;**23**:1801–6. <https://doi.org/10.1093/bioinformatics/btm233>
- Jobson RW, Albert VA. Molecular rates parallel diversification contrasts between carnivorous plant sister lineages. *Cladistics* 2002;**18**:127–36. <https://doi.org/10.1111/j.1096-0031.2002.tb00145.x>
- Kearse M, Moir R, Wilson A *et al.* Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. *Bioinformatics* 2012;**28**:1647–9. <https://doi.org/10.1093/bioinformatics/bts199>
- Kobrová L, Hroneš M. First insights into the evolution of genome size in the borage family: a complete data set for Boraginaceae from the Czech Republic. *Botanical Journal of the Linnean Society* 2019;**189**:115–31. <https://doi.org/10.1093/botlinnean/boy079>
- Kolde R. *pheatmap: Pretty Heatmaps*. R package version 1.0.12, 2018. <https://github.com/raivokolde/pheatmap>
- Krahulcová A, Jarolímová V. Relationship between *Pinguicula bohemica* Krajina and *Pinguicula vulgaris* L. (Lentibulariaceae) from the karyological point of view. *Preslia* 1991;**53**:323–8.
- Löve A, Kjellqvist E. Cytotaxonomy of Spanish plants. IV. Dicotyledons: Caesalpiniaceae–Asteraceae. *Lagascalia* 1974;**4**:153–211.
- Lustofin K, Świątek P, Stolarczyk P *et al.* Do food trichomes occur in *Pinguicula* (Lentibulariaceae) flowers? *Annals of Botany* 2020;**126**:1039–48. <https://doi.org/10.1093/aob/mcaa123>
- Lynch M, Conery JS. The origins of genome complexity. *Science* 2003;**302**:1401–4.
- Majesky L, Hroneš M, Kitner M *et al.* *Pinguicula vulgaris* in central Europe: When does one species turn into another? *Preslia* 2022;**94**:275–304. <https://doi.org/10.23855/preslia.2022.275>
- Mandáková T, Pouch M, Harmanová K *et al.* Multispeed genome diploidization and diversification after an ancient allopolyploidization. *Molecular Ecology* 2017;**26**:6445–62. <https://doi.org/10.1111/mec.14379>
- Moore DM. *Flora Europaea Check-List and Chromosome Index*. Cambridge: Cambridge University Press, 1982.
- Murin A. Substitution of cellophane for glass covers to facilitate preparation of permanent squashes and smears. *Stain Technology* 1960;**35**:351–3.
- Pavlovič A, Koller J, Vrobel O *et al.* Is the co-option of jasmonate signalling for botanical carnivory a universal trait for all carnivorous plants? *Journal of Experimental Botany* 2024;**75**:334–49. <https://doi.org/10.1093/jxb/era359>
- Pérez AJ, Tobar F, Burgess KS *et al.* Contributions to Ecuadorian butterworts (Lentibulariaceae, *Pinguicula*): two new species and a re-evaluation of *Pinguicula calyptata*. *PhytoKeys* 2023;**222**:153–71. <https://doi.org/10.3897/phytokeys.222.98139>
- Perrier X, Jacquemoud-Collet JP. DARwin software, 2006. <http://darwin.cirad.fr/darwin>
- Peruzzi L, Gestri G. A new butterwort species (*Pinguicula*, Lentibulariaceae) from Northern Apennine (Italy). *Plant Biosystems—An International Journal Dealing with All Aspects of Plant Biology* 2013;**147**:692–703. <https://doi.org/10.1080/11263504.2012.756073>
- Roccia A. *Pinguicula x gresivaudanica* (Lentibulariaceae), a new butterwort hybrid from the French Alps. *Carnivorous Plant Newsletter* 2013;**42**:36–46. <https://doi.org/10.55360/cpn422.ar445>
- Roccia A, Gluch O, Lampard S *et al.* *Pinguicula of the Temperate North*. Poole: Redfern Natural History, 2016.
- Ronquist F, Teslenko M, Van der Mark P *et al.* MrBayes 3.2: Efficient Bayesian phylogenetic inference and model choice across a large model space. *Systematic Biology* 2012;**61**:539–42. <https://doi.org/10.1093/sysbio/sys029>
- Rosenberg NA. DISTRUCT: a program for the graphical display of population structure. *Molecular Ecology Notes* 2004;**4**:137–8. <https://doi.org/10.1046/j.1471-8286.2003.00566.x>
- Schlauer J. ERRATUM: The correct name for the hybrid butterwort *Pinguicula grandiflora* subsp. *rosea* 'P. vulgaris'. *Carnivorous Plant Newsletter* 2013;**42**:93. <https://doi.org/10.55360/cpn423.js759>
- Schluter PM, Harris SA. Analysis of multilocus fingerprinting data sets containing missing data. *Molecular Ecology Notes* 2006;**6**:569–72. <https://doi.org/10.1111/j.1471-8286.2006.01225.x>
- Shimai H, Setoguchi H, Roberts DL, *et al.* Biogeographical patterns and speciation of the genus *Pinguicula* (Lentibulariaceae) inferred by phylogenetic analyses. *PLoS One* 2021;**16**:e0252581. <https://doi.org/10.1371/journal.pone.0252581>
- Silva SR, Gibson R, Adamec L *et al.* Molecular phylogeny of bladderworts: A wide approach of *Utricularia* (Lentibulariaceae) species relationships based on six plastidial and nuclear DNA sequences. *Molecular Phylogenetics and Evolution* 2018;**118**:244–64. <https://doi.org/10.1016/j.ympev.2017.10.010>
- Silva SR, Diaz YC, Penha HA, *et al.* The chloroplast genome of *Utricularia reniformis* sheds light on the evolution of the *ndh* gene complex of terrestrial carnivorous plants from the Lentibulariaceae family. *PLoS One* 2016;**11**:e0165176. <https://doi.org/10.1371/journal.pone.0165176>
- Šmarda P, Knapke O, Březinová A *et al.* Genome sizes and genomic guanine + cytosine (GC) contents of the Czech vascular flora with new estimates for 1700 species. *Preslia* 2019;**91**:117–42. <https://doi.org/10.23855/preslia.2019.117>
- Steiger J. *Pinguicula* (Lentibulariaceae): the cool climate species of the northern hemisphere—morphology, biology, cultivation. In: Schlauer J, Meyers-Rice B (eds.), Second Conference of the International Carnivorous Plant Society, Bonn, Germany, 1998, pp. 12–3.
- Stevens PF. Angiosperm Phylogeny Website, version 14, July 2017, last updated 14 October 2023, 2001. <http://www.mobot.org/MOBOT/research/APweb/> (1 March 2024, date last accessed).
- Suda J, Krahulcová A, Trávník P *et al.* Ploidy level versus DNA ploidy level: an appeal for consistent terminology. *Taxon* 2006;**55**:447–50. <https://doi.org/10.2307/25065591>
- Tamura K, Stecher G, Kumar S. MEGA11: molecular evolutionary genetics analysis version 11. *Molecular Biology and Evolution* 2021;**38**:3022–7. <https://doi.org/10.1093/molbev/msab120>
- Uchiyama H. Chromosome number of *Pinguicula vulgaris* var. *macroceras* from Japan. *Chromosome Information Service (CIS)* 1990;**49**:24–5.
- Veleba A, Bureš P, Adamec L *et al.* Genome size and genomic GC content evolution in the miniature genome-sized family Lentibulariaceae. *New Phytologist* 2014;**203**:22–8. <https://doi.org/10.1111/nph.12790>
- Vos P, Rogers R, Bleeker M *et al.* AFLP—a new technique for DNA-fingerprinting. *Nucleic Acids Research* 1995;**23**:4407–14. <https://doi.org/10.1093/nar/23.21.4407>
- Wang X, Morton JA, Pellicer J *et al.* Genome downsizing after polyploidy: mechanisms, rates and selection pressures. *The Plant Journal* 2021;**107**:1003–15. <https://doi.org/10.1111/tpj.15363>
- Wicke S, Schäferhoff B, de Pamphilis CW *et al.* Disproportional plastome-wide increase of substitution rates and relaxed purifying selection in genes of carnivorous Lentibulariaceae. *Molecular Biology and Evolution* 2014;**31**:529–45. <https://doi.org/10.1093/molbev/mst261>
- Yoshimura K. Studies on the chromosome number and karyotype of *P. ramosa* (Lentibulariaceae). *The Journal of Japanese Botany* 1973;**48**:289–94.
- Zamora R, Jamilena M, Ruiz-Rejón MR *et al.* Two new species of the carnivorous genus *Pinguicula* (Lentibulariaceae) from Mediterranean habitats. *Plant Systematics and Evolution* 1996;**200**:41–60. <https://doi.org/10.1007/BF00984747>