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Reticulate phylogenetics and phytogeographical structure of *Heliosperma* (*Sileneae*, Caryophyllaceae) inferred from chloroplast and nuclear DNA sequences

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Abstract

The Balkan Peninsula is known to be one of the most diverse and species-rich parts of Europe, but its biota has gained much less attention in phylogenetic and evolutionary studies compared to other southern European mountain systems. We used nuclear ribosomal internal transcribed spacer (ITS) sequences and intron sequences of the chloroplast gene *rps16* to examine phylogenetic and biogeographical patterns within the genus *Heliosperma* (*Sileneae*, Caryophyllaceae). The ITS and *rps16* intron sequences both support monophyly of *Heliosperma*, but the data are not conclusive with regard to its exact origin. Three strongly supported clades are found in both data sets, corresponding to *Heliosperma alpestre*, *Heliosperma macranthum* and the *Heliosperma pusillum* clade, including all other taxa. The interrelationships among these three differ between the nuclear and the plastid data sets. Hierarchical relationships within the *H. pusillum* clade are poorly resolved by the ITS data, but the *rps16* intron sequences form two well-supported clades which are geographically, rather than taxonomically, correlated. A similar geographical structure is found in the ITS data, when analyzed with the NeighbourNet method. The apparent rate of change within *Heliosperma* is slightly higher for *rps16* as compared to ITS. In contrast, in the *Sileneae* outgroup, ITS substitution rates are more than twice as high as those for *rps16*, a situation more in agreement with what has been found in other rate comparisons of noncoding cpDNA and ITS. Unlike most other *Sileneae* ITS sequences, the *H. pusillum* group sequences display extensive polymorphism. A possible explanation to these patterns is extensive hybridization and gene flow within *Heliosperma*, which together with concerted evolution may have eradicated the ancient divergence suggested by the *rps16* data. The morphological differentiation into high elevation, mainly widely distributed taxa, and low elevation narrow endemics is not correlated with the molecular data, and is possibly a result of ecological differentiation.

Keywords: *Heliosperma*; *Ixoca*; Balkan biogeography; ITS; *rps16*; Reticulate evolution

1. Introduction

The evolution and diversification of plants in the southern European mountain systems since their uplift 10–2 million years ago (Ager, 1975) have been strongly associated with climatic changes during the Pleistocene (Merxmüller, 1952; Kadereit et al., 2004). Several surveys have been carried out during the past years to reveal phylogeographic patterns influenced by the glaciations as well as to test previous biogeographic hypotheses (e.g. Comes and Kadereit, 1998; Stehlik et al., 2002; Schönswetter et al., 2005). The main focus has been on the Alps, whereas other Southern European mountain systems have gained less attention (but see e.g. Gutierrez Larena et al., 2002; Schönswetter et al., 2003; Vargas, 2003; Lihova et al., 2004). The Balkan Peninsula has been almost completely neglected or only included as a part of studies performed with a broader geographic scope (Trewick et al., 2002; Hampe et al., 2003;

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Comes and Kadereit, 2003; Vargas, 2003; Lascoux et al., 2004). An exception is an isozyme survey of Balkan beech populations that showed a north-west to south-east cline in genetic differentiation (Gömöry et al., 1999). However, no phylogeographic study on herbaceous plants from the Balkan Peninsula has been published and the biodiversity patterns and their underlying processes remain poorly documented and understood (Kryštufek and Reed, 2004).

The Balkan Peninsula has long been known for its high level of biodiversity and the Balkan flora is thought to be the richest in Europe (Turrill, 1929; Polunin, 1997; Kryštufek and Reed, 2004). The reasons for this diversity are the geographic position of the Balkans at the transition of different floral provinces, topographic, climatic and geological complexity, and the relatively high environmental stability through geologic history (Polunin, 1997; Reed et al., 2004). The Balkan Peninsula was an important refugial area for plants and animals during the Pleistocene (e.g. Comes and Kadereit, 1998; Hewitt, 2000; Hampe et al., 2003; Petit et al., 2003; Eastwood, 2004; Tzedakis, 2004). The Dinaric mountains were, compared to other Southern European mountain systems, much less affected by glaciations. Only the highest peaks were glaciated and there was no continuous ice sheet present as in the Alps or Pyrenees (Turrill, 1929). The snow line was at approximately 1500 m above sea level, which is only 1000 m lower than today and the diversity as well as altitudinal differentiation of habitats was therefore greater than in other Southern European mountain systems (Turrill, 1929; Horvat et al., 1974).

Climatic oscillations during the Quaternary period triggered latitudinal and altitudinal migrations and (local) extinctions of animals and plants, i.e. distributional range contractions and fragmentations as well as subsequent range expansions due to climatic changes during glacials and interglacials (Comes and Kadereit, 1998; Hewitt, 2000; Petit et al., 2003). Secondary contacts between previously isolated populations may have led to sympatric speciation via hybridization and polyploidization (Stebbins, 1984). In general, hybridization has played an important role in plant evolutionary histories and has often resulted in the occurrence of new hybrid lineages, both at the polyploid and homoploid level (reviewed by e.g. Rieseberg and Wendel, 1993; Rieseberg and Carney, 1998; Soltis et al., 2003). Discordances between different gene trees (mostly chloroplast and nuclear ribosomal genes), or alternatively, between gene tree(s) and morphology, have often been explained as a result of hybridization (e.g. Soltis and Kuzoff, 1995; Wolf et al., 1997; McKinnon et al., 2001; Doyle et al., 2003; Okuyama et al., 2005). Lineage sorting, i.e. random extinction of ancestral alleles or paralogues, is an alternative but less favored explanation. Additive polymorphic patterns in different nuclear markers (e.g. Oxelman, 1996; Whittall et al., 2000; Andreasen and Baldwin, 2003; Fuertes Aguilar and Nieto Feliner, 2003; Franzke et al., 2004) and the application of low copy nuclear gene trees (e.g. Popp and Oxelman, 2001; Doyle et al., 2003; Popp et al., 2005) have improved the potential to understand reticulate evolution.

In order to examine the role of different evolutionary processes in the formation of Balkan biodiversity as well as to reveal possible phylogeographic patterns in the Balkan Peninsula, we have used chloroplast and nuclear DNA sequences from the genus *Heliosperma* (Rchb.) Rchb. (=*Ixoca* Rafin.), nom. cons. prop (Frajman and Rabeler, 2006), tribe *Sileneae* (Caryophyllaceae). Most of the *Heliosperma* taxa are endemic to relatively small areas of the Balkan Peninsula, but *H. alpestre* grows in the Alps, and *H. pusillum* is more widespread, distributed in the southern European mountains from the Sierra Cantabrica to the Carpathians (Chater et al., 1993; Jalas and Suominen, 1986). They are caespitose perennial herbs with slender, branched stems up to 30 cm high and usually grow in damp, open habitats, often on calcareous rocks. The variation in habit, leaf-shape and hairiness shows some geographical and ecological correlation, which has been variously interpreted by taxonomists in the past. The position of *Heliosperma* within *Sileneae* has been unstable throughout the taxonomic history (reviewed by Frajman and Rabeler, 2006).

As far as the interspecific relationships are concerned, no recent revisionary work has been done on *Heliosperma* as a whole. The most comprehensive study was performed by Neumayer (1923, 1924), who recognized only three species: *Silene macrantha* (Pančić) Neum. (≡*H. macranthum*), *Silene alpestris* Jacq. (≡*H. alpestre*, *H. quadrifidum* (L.) Griseb., nom. utique. rej. prop. (Frajman, in press)) and *Silene quadridentata* (L.) Pers. sensu lato (≡*H. pusillum*), the latter including 16 subspecific taxa. Other botanists have recognized from 4 to 16 species with several infraspecific taxa (Ascherson and Graebner, 1920; Chowduri, 1957; Trinajstić, 1979; Greuter et al., 1984; Ikonnikov, 1984; Chater et al., 1993). Endemic *Heliosperma* taxa from the Balkan Peninsula were recently revised by Niketić and Stevanović (in press). In the following test, we follow a taxonomy combined from Trinajstić (1979), Chater et al. (1993), and Niketić and Stevanović (in press).

Morphologically, *H. alpestre* and *H. macranthum* are very distinct and different from all other taxa (e.g. Neumayer, 1924). However, *H. pusillum* sensu lato is highly variable and two groups of taxa can be recognized applying morphological and ecological criteria (Ascherson and Graebner, 1920; Neumayer, 1923; Trinajstić, 1979; personal observations). The low elevation group includes narrow endemics of the Balkan Peninsula, growing mainly in canyons and gorges, often under cliff overhangs at lower elevations, and in southern Balkans also at higher elevations: *Heliosperma veselskyi* (Slovenia), *Heliosperma retzdorffianum* (Bosnia and Herzegovina), *Heliosperma insulare* (Croatia), *Heliosperma oliverae* (Montenegro), *Heliosperma tommasinii* (Montenegro, Albania), *Heliosperma nikolicii* (Kosovo), *Heliosperma vandasii* (Macedonia) and *Heliosperma intonsum* and *Heliosperma chromodontum* (Greece). They are usually densely hairy, with long

multicellular glandular hairs, and their leaves are broader and the stem internodes shorter compared to the other group. The papillae on the seed crest usually do not exceed one fourth of the seed diameter. The other group (high elevation group) is composed of taxa from higher elevations, which are both less morphologically distinct and have broader distributions, which are often overlapping. They mostly grow above the timberline, occasionally also on cliffs, river banks and gravely sites at lower elevations. They are mostly glabrous or sparsely hairy, often with unicellular glands, much more slender in habit and have longer papillae on the dorsal seed crest. Most of these characteristics are shared with *H. macranthum* and *H. alpestre* (Neumayer, 1923), and could therefore possibly be interpreted as plesiomorphic. The high elevation group includes the widely distributed *H. pusillum* sensu stricto as well as other taxa, often thought to be conspecific with *H. pusillum: Heliosperma pudibundum*, *Heliosperma monachorum* and *Heliosperma albanicum*. Neumayer (1923) noted that in some cases there are transitional forms between the two groups, possibly due to the fact that hybridization might occur between low and high elevation taxa. It is possible to hypothesize independent origins of the low and high elevation groups, thus assuming that they are both monophyletic. Alternatively, either the low- or the highland group may have been derived from the other group, or the morphologically distinct lowland endemics have arisen independently from each other, and the morphological differentiation from the highland group rather reflects parallel adaptations to the chasmophytic habitat.

Using DNA intron sequences from the chloroplast gene *rps16* and nuclear ribosomal internal transcribed spacer (ITS) regions, we examine phylogenetic and biogeographic patterns within *Heliosperma* to investigate the role of different evolutionary processes in the formation of the Balkan biodiversity. For both these regions, there is a large amount of data available in *Sileneae* already (Oxelman and Lidén, 1995; Oxelman et al., 1997, 2001; Popp and Oxelman, 2004), which have proven phylogenetically informative. The *rps16* region has also proven useful in several other phylogenetic studies (see e.g. Shaw et al., 2005). In particular, we examine whether the differentiation of *H. pusillum* sensu lato into low and high elevation forms is due to phylogenetic or ecological differentiation and we examine the hypothesis that *H. alpestre* and *H. macranthum* are phylogenetically distinct from all other *Heliosperma* taxa. We also test the monophyly of *Heliosperma* and explore its position within *Sileneae*.

2. Material and methods

2.1. Plant material and DNA extraction

We sampled all *Heliosperma* taxa ever recognized at the specific level, except *Heliosperma arcanum* Zapal. (which is most likely extinct), as well as several infraspecific taxa. Specimens were determined using national as well as regional floras (e.g. Trinajstić, 1979) and some were sampled from *loci classici*. From widely distributed species, like *H. pusillum*, several accessions from different parts of the distribution area were sequenced. One individual per population was sequenced, with exception being HnikSR1 and HnikSR2, as well as HpusSLO4 and HpusSLO5, pairs which represent one population each. The selection of outgroup taxa was based on previous studies (Oxelman and Lidén, 1995; Oxelman et al., 1997; Popp and Oxelman, 2004).

Total genomic DNA was extracted from herbarium specimens or silica-gel dried material following the protocol described by Oxelman et al. (1997) and purified using the QIAquick purification kit protocol (QiaGen). Voucher data and EMBL/GenBank accession numbers of *Heliosperma* specimens used in the study are presented in Table 1. Data for outgroup taxa included in the analyses are listed by Popp and Oxelman (2004).

2.2. PCR, sequencing, contig assembly and polymorphic sites

PCRs were performed as described by Popp and Oxelman (2004). Some PCRs were performed using the Phusion™ High-Fidelity PCR Master Mix according to the manufacturer's instructions (Finnzymes).

The ITS and the *rps16* regions were amplified using the primer pairs P17/26S-82R (Popp and Oxelman, 2001) and rpsF/rpsR2 (Oxelman et al., 1997), respectively. PCR products were purified using Multiscreen PCR (Millipore) according to the manufacturer's protocol and then sequenced with the nested primers P16b/ITS4 (Popp et al., 2005; White et al., 1990) for ITS and rpsF2a/rpsR3R (Popp et al., 2005) for *rps16*, using the DYEnamic ET Terminator Cycle Sequencing Premix Kit (Amersham Pharmacia Biotech) and visualized on a MEGABace 1000 DNA Analysis System (Amersham Pharmacia Biotech), or the BigDye® Terminator Cycle Sequencing Kit (Applied Biosystems) and visualized on ABI 3700 or ABI3730XL (Applied Biosystems).

Contigs were assembled and edited using Sequencher 3.1.1 (Gene Codes Corporation). Base polymorphisms were coded using the NC-IUPAC ambiguity codes. A polymorphism was coded when more than one peak was present at the same position in the electropherogram. If a double signal was present in both strands (forward and reverse), the polymorphisms were scored when the weakest signal reached at least one third of the height of the strongest signal in both strands. In some cases, we did not obtain sequence information from both strands. Sites with double signal were in such cases scored as polymorphic when the weakest signal reached at least half of the height of the strongest signal. As we aimed to identify individuals containing different ITS copies that might result from hybridization of two homozygous parental ribotypes, we focused on additive polymorphic sites (APS) within *Heliosperma* (Table 2). These were recorded in cases when the

Table 1
Acronyms (as used on the trees), voucher data and GenBank accession numbers for specimens used in this study

Acronym	Locality and voucher	GenBank Accession No. (*rps16*, ITS)
Ingroup taxa		
HalbGR		
H. albanicum K. Maly	Greece: Smolikas Mts.; W 1996-07664	/, EF118060
HalbMC		
H. albanicum K. Maly	Macedonia: Šarplanina Mts., Popova Šapka; LJU 50100	EF118112, EF118081
HalbMN		
H. albanicum K. Maly	Montenegro: Komovi Mts.; LJU 136608	EF118114, EF118089
HalbSR		
H. albanicum K. Maly	Serbia: Šarplanina Mts., Durlov potok; LJU 135671	EF118113, EF118073
HalpSLO1		
H. alpestre (Jacq.) Griseb.	Slovenia: Kamniške Alps, Češka koča hut;LJU 135843	EF118117, EF118056
HalpSLO2		
H. alpestre (Jacq.) Griseb.	Slovenia: Primorska, Mt. Snežnik; LJU 136562	EF118116, EF118055
HalpSLO3		
H. alpestre (Jacq.) Griseb.	Slovenia: Julian Alps, Vrata Valley; LJU 136278	EF118115, EF118054
HcanMC		
Silene pusilla Waldst. et Kit. subsp. *candavica* (H. Neumayer) Greuter & Burdet	Macedonia: Mt. Jablanica; LJU 137734	EF118118, EF118061
HchrGR1		
H. chromodontum (Boiss. Et Reuter) Juratzka	Greece: Thessalia, Olimbos; LJU 46012	EF118120, EF118097
HchrGR2		
H. chromodontum (Boiss. Et Reuter) Juratzka	Greece: Thessalia, Olimbos; LJU 100055	EF118119, EF118096
HinsCRO		
H. insulare Trinajstić	Croatia: Mljet island, Mt. Veliki Grad; LJU 136561	EF118121, EF118074
HintGR		
H. intonsum (Melzh. & Greuter) Niketić & Stevanović	Greece: Aoos gorge, 2 km S of Konitsa; A. Strid reference herbarium No. 9058	EF118122, EF118075
HmacAL		
H. macranthum Pančić	Albania: Malesia e Madhe, Mt. Bjeshket e Namuna; LJU 136592	EF118123, EF118057
HmacMN1		
H. macranthum Pančić	Montenegro: Prokletije Mts., Ćafa Borit; LJU 137735	EF118125, EF118059
HmacMN2		
H. macranthum Pančić	Montenegro: Komovi Mts.; LJU 136611	EF118124, EF118058
HmonBIH1		
H. monachorum Vis. & Pančić	Bosnia & Herzegovina: Plužine, Zalomska valley; LJU 136580	EF118126, EF118076
HmonBIH2		
H. monachorum Vis. & Pančić	Bosnia & Herzegovina: Rogatica, Banja Sijena; LJU 136606	EF118129, EF118079
HmonSR1		
H. monachorum Vis. & Pančić	Serbia: Peručac, Bajina Bašta; LJU 39516	EF118127, EF118077
HmonSR2		
H. monachorum Vis. & Pančić	Serbia: Tara Mts.; LJU 136569	EF118128, EF118078
HnikAL		
H. nikolicii (Seliger & T. Wraber) Niketić & Stevanović	Albania: Mt. Jalice (Đalićes), SkalaBicaj; LJU 137736	EF118131, EF118063
HnikSR1		
H. nikolicii (Seliger & T. Wraber) Niketić & Stevanović	Serbia: Kosovo, Prizrenska Bistrica gorge; LJU 100080	EF118130, EF118091
HnikSR2		
H. nikolicii (Seliger & T. Wraber) Niketić & Stevanović	Serbia: Kosovo, Prizrenska Bistrica gorge; LJU 137737	EF118132, EF118062
HoliMN		
H. oliverae Niketić & Stevanović	Montenegro: Prokletije Mts., Maja Šćapica; LJU 137738	EF118133, EF118064
HpudA		
H. pudibundum (Hoffmanns.) Griseb.	Austria: Hohe Tauern, Mt. Wandspitze; LJU 137740	EF118134, EF118065
HpusA		
H. pusillum (Waldst. & Kit.) Rchb.	Austria: Karnische Alpen, Kirchbach, Straniger Alm; LJU 137741	EF118151, EF118106
HpusB		
H. pusillum (Waldst. & Kit.) Rchb.	Bulgaria: Mt. Pirin; S (Antonoff, 1930)	EF118139, EF118084
HpusBIH1		
H. pusillum (Waldst. & Kit.) Rchb.	Bosnia & Herzegovina: Sutjeska valley, Vratar; LJU 136568	EF118135, EF118080
HpusBIH2		
H. pusillum (Waldst. & Kit.) Rchb.	Bosnia & Herzegovina: Treskavica Mts.; LJU 137742	EF118154, EF118068
HpusBIH3		

(*continued on next page*)

Table 1 (*continued*)

Acronym	Locality and voucher	GenBank Accession No. (*rps16*, ITS)
H. pusillum (Waldst. & Kit.) Rchb. *HpusBIH4*	Bosnia & Herzegovina: Mt. Maglič; LJU 137743	EF118156, EF118070
H. pusillum (Waldst. & Kit.) Rchb. *HpusCH*	Bosnia & Herzegovina: Zelengora Mts.; LJU 137744	EF118157, EF118071
H. pusillum (Waldst. & Kit.) Rchb. *HpusCRO*	Switzerland: above Kandergrund; Z 1438	EF118150, EF118066
H. pusillum (Waldst. & Kit.) Rchb. *HpusF1*	Croatia: Velebit Mts., Vaganski vrh; LJU 136567	EF118136, EF118098
H. pusillum (Waldst. & Kit.) Rchb. *HpusF2*	France: Pyrenees, Eaux Bonnes; S (Neyraut, 1906)	EF118140, EF118104
H. pusillum (Waldst. & Kit.) Rchb. *HpusmarkAL*	France: Pyrenees, La Pierre St Martin; ZT 791	EF118149, EF118105
H. pusillum (Waldst. & Kit.) Rchb. subsp. *markgrafii* (Neumayer) *HpusMN*	Albania: Mt. Paštrik; LJU 136591	EF118158, EF118090
H. pusillum (Waldst. & Kit.) Rchb. *HpusPL*	Montenegro: Prokletije Mts., Grebaja; LJU 136593	EF118142, EF118082
H. pusillum (Waldst. & Kit.) Rchb. *HpusRO1*	Poland: Carpathians, Zakopane; S (Nilsson & Degelius, 1929)	EF118141, EF118088
H. pusillum (Waldst. & Kit.) Rchb. *HpusRO2*	Romania: Carpathians, Mt. Fagaraš; LJU 136612	EF118147, EF118085
H. pusillum (Waldst. & Kit.) Rchb. *HpusSK*	Romania: Carpathians, Postavaru; LJU 136610	EF118148, EF118086
H. pusillum (Waldst. & Kit.) Rchb. *HpusSLO1*	Slovakia: High Tatre Mts.; LJU 137745	EF118155, EF118069
H. pusillum (Waldst. & Kit.) Rchb. *HpusSLO2*	Slovenia: Kamniške Alps, Češka koča hut; LJU 136277	EF118144, EF118102
H. pusillum (Waldst. & Kit.) Rchb. *HpusSLO3*	Slovenia: Julian Alps, Malo Polje; LJU 135842	EF118145, EF118103
H. pusillum (Waldst. & Kit.) Rchb. *HpusSLO4*	Slovenia: Trnovski gozd, lednica v Paradani; LJU 136557	EF118138, EF118101
H. pusillum (Waldst. & Kit.) Rchb. *HpusSLO5*	Slovenia: Primorska, Mt. Snežnik, Kosmata dolina; LJU 136566	EF118137, EF118099
H. pusillum (Waldst. & Kit.) Rchb. *HpusSR1*	Slovenia: Primorska, Mt. Snežnik, Ilovca; LJU 136564	EF118146, EF118100
H. pusillum (Waldst. & Kit.) Rchb. *HpusSR2*	Serbia: Mt. Rtanj; LJU 136570	EF118143, EF118083
H. pusillum (Waldst. & Kit.) Rchb. *HpusUA*	Serbia: Stara planina Mts, Babin zub; LJU 137746	EF118153, EF118067
H. pusillum (Waldst. & Kit.) Rchb. *HretBIH*	Ukraine: Carpathians, Cornogora; LWKS 5962	EF118152, EF118087
H. retzdorffianum K. Maly *HtomMN1*	Bosnia & Herzegovina: Jablanica; LJU 136559	EF118159, EF118092
H. tommasinii (Vis.) Rchb. *HtomMN2*	Montenegro: Rumija Mts., Lonac (Sutorman); LJU 136560	EF118160, EF118094
H. tommasinii (Vis.) Rchb. *HvanMC*	Montenegro: Lovčen Mts.; LJU 136613	EF118161, EF118093
H. chromodontum (Boiss. et Reuter) Juratzka subsp. *Vandasii* (Neumayer) Trinajstić *HvesgluSLO*	Macedonia: Galičica Mts., Stenje; S (Joško, 1930)	EF118162, EF118095
H. veselskyi Janka subsp. *glutinosum* (Zois in Juratzka) E. Mayer *HvesvesSLO1*	Slovenia: Gorenjska, Medvode, above Zbiljsko jezero; LJU 136287	EF118163, EF118107
H. veselskyi Janka subsp. *veselskyi* *HvesvesSLO2*	Slovenia: Ljubljana, Iški vintgar, Krvavice; LJU 136286	EF118164, EF118109
H. veselskyi Janka subsp. *veselskyi* *HvesvesSLO3*	Slovenia: Zasavje, Trbovlje, Mitoviški slap; LJU 136284	EF118165, EF118108
H. veselskyi Janka subsp. *veselskyi* *HveswidSLO*	Slovenia: Primorska, Trebuša valley, Mt. Kobilica; LJU 93689	EF118166, EF118110
H. veselskyi Janka subsp. *widderi* (Kofol-Seliger & T. Wraber) Trpin & Vreš	Slovenia: Koroška, Mučka Bistrica valley; LJU 136285	EF118167, EF118072

Table 1 (*continued*)

Acronym	Locality and voucher	GenBank Accession No. (*rps16*, ITS)
Outgroup taxa		
Agrostemma githago L.	Popp & Oxelman, 2004	Z83154, X86895
Atocion armeria Fourr.	Popp & Oxelman, 2004	Z83159, X86880
Atocion lerchenfeldianum (Baumg.) M. Popp	Popp & Oxelman, 2004	Z831061, X868057
Atocion rupestre (L.) B. Oxelman	Popp & Oxelman, 2004	Z83160, X86874
Eudianthe coeli-rosa (L.) Endl.	Popp & Oxelman, 2004	Z83156, X86881
Eudianthe laeta (Aiton) Rchb. ex Willk.	Popp & Oxelman, 2004	Z83155, X86882
Lychnis abyssinica (Hochst) M. Lidén	Popp & Oxelman, 2004	Z83161,X86890
Lychnis chalcedonica L.	Popp & Oxelman, 2004	Z83164, X86894
Lychnis coronaria Desr.	Popp & Oxelman, 2004	Z83165, X86891
Lychnis flos-cuculi L.	Popp & Oxelman, 2004	Z83163, X86893
Lychnis flos-jovis Desr.	Popp & Oxelman, 2004	Z83166, X86892
Petrocoptis pyrenaica A. Br.	Popp & Oxelman, 2004	Z83167, X86875
Silene acaulis (L.) Jacq.	Popp & Oxelman, 2004	Z83189, X86860
Silene baccifera (L.) Roth	Popp & Oxelman, 2004	Z83169, X86889
Silene bergiana Lindman	Popp & Oxelman, 2004	Z83191,X86835
Silene conica L.	Popp & Oxelman, 2004	Z83170, X86832
Silene fruticosa L.	Popp & Oxelman, 2004	Z83188, X86865
Silene linnaeana Voroschilov	Popp & Oxelman, 2004	Z831060, X868058
Silene nivalis Rohrb.	Popp & Oxelman, 2004	Z83190, X86861
Silene noctiflora L.	Popp & Oxelman, 2004	Z83176, X86829
Silene nocturna L.	Popp & Oxelman, 2004	Z83192, X86841
Silene rotundifolia Nutt.	Popp & Oxelman, 2004	Z83183, X86887
Silene schafta S.G.Gmel. ex Hohen.	Popp & Oxelman, 2004	Z83194, X86852
Viscaria alpina (L.) G. Don.	Norway: Møre og Romsdal, Sunnmørsalpane; Herbarium Schönswetter & Tribsch 10906	EF118168, EF118111
Viscaria vulgaris Rohl.	Popp & Oxelman, 2004	Z831912, X868911

Herbarium acronyms are according to Holmgren and Holmgren (1998).

two bases involved in a polymorphic site were also found separately in other accessions in the data set, following Fuertes Aguilar and Nieto Feliner (2003). We explored the distribution of APS by dividing the *H. pusillum* group accessions into five geographic regions, based on the *rps16* phylogenies and the ITS split network (Fig. 1). Ancestral states for the parsimony informative APS sites were inferred using Mesquite version 1.06 (Maddison and Maddison, 2005), with the ''Trace Character Over Trees'' module applying the parsimony reconstruction method over all most parsimonious ITS trees. This module summarizes what ancestral states are reconstructed for the clades with identical taxon content in a series of trees.

The sequences were aligned manually, using Se-Al Ver. 1.0a1 (Rambaut, 1996) and BioEdit (Hall, 1999), using the criteria described by Popp and Oxelman (2004). Gaps (indels) were coded to binary characters using SeqState version 1.25 (Müller, 2005). Alignments are available upon request from the first author.

2.3. Phylogenetic analyses

Maximum parsimony (MP) analyses as well as MP bootstrap analyses of ITS and *rps16* data sets were performed using PAUP* version 4.0b10 for Windows (Swofford, 2002). The most parsimonious *rps16* trees were searched for heuristically with 1000 replicates of random sequence addition, TBR swapping, MULPARS on, and a maximum of 1000 trees saved per replicate (nchuck = 1000), whereas the most parsimonious ITS trees were searched for heuristically with 100,000 replicates of random sequence addition, TBR swapping, MULPARS off, and DELTRAN optimization. All characters were equally weighted and unordered. The data were bootstrapped using full heuristics, 1000 replicates, TBR branch swapping, MULTREES option off, and random addition sequence with five replicates. *Agrostemma githago* was used as outgroup for rooting, based on previous studies (Oxelman and Lidén, 1995; Oxelman et al., 1997). The average Jukes Cantor (JC) corrected pairwise distances for in- and outgroup were compared, in order to quantify the relative rates of substitutions.

Bayesian analysis was performed on a limited data set including only *Heliosperma* taxa, using MrBayes version 3.1 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003). The data was partitioned into a nucleotide set and an indel set, which was treated as morphological data according to the model of Lewis (2001). Each data

Table 2
Parsimony informative positions of the *H. pusillum* group in the ITS alignment containing additive polymorphic sites (APS) and indels (653, 681), indicating hybridization among populations from different geographic groups (Fig. 4)

Geographic group	Position in ITS matrix																					
	66	67	95	112	166	180	187	195	205	439	440	441	446	515	517	565	579	582	586	589	653	681
P/A (6 accessions)	A	A	C	G	**C** (2) T (3) **Y** (1)	A	**T** (4) **W** (2)	**C** (2) T (4)	**A** (5) **M** (1)	**A** (4) **M** (2)	T	G	G	C	T	G	A	C (4) **M** (2)	C	**C** (5) **Y** (1)	0	**0** (3) 1 (3)
A/D (10 accessions)	A	A	C	G (9) **T** (1)	T	T (9) **W** (1)	A (7) **W** (3)	T	**A** (3) C (2) **M** (5)	**A** (3) C (1) **M** (6)	T	G (9) K (1)	G	C	T	G	A	C (6) **M** (4)	C	**C** (7) **Y** (2) **H** (1)	0 (7) 1 (3)	**0**
D (4 accessions)	A (1) R (3)	A (1) M (3)	C	G (1) **T** (1) **K** (2)	T	T	A	**C** (1) T (1) **Y** (2)	C (3) **Y** (1)	C (2) **M** (2)	G (2) K (2)	T (2) K (2)	G (3) R (1)	C	**T** (1) G (1) **K** (2)	**G** (1) A (1) **R** (2)	A (1) C (1) **M** (2)	C (1) T (1) **Y** (2)	C (1) T (1) **Y** (2)	**C** (1) T (1) **Y** (2)	0 (3) 1 (1)	1
D/R (26 accessions)	A (10) G (5) R (11)	A (9) C (6) M (6) Y (2) W (2) H (1)	C (19) A (3) M(4)	G (25) **T** (1)	**C** (1) T (24) **Y** (1)	T	A	**C** (3) T (21) **Y** (2)	C (22) **M** (1) **Y** (2) −(1)	C (25) **M** (1)	T (4) G (6) C (2) K (11) Y (3)	G (8) T (6) K (12)	G (9) A (6) R (11)	C	**T** (13) G (4) **K** (9)	**G** (11) A (5) **R** (10)	A (13) C (4) **M** (9)	C (20) T (2) **Y** (4)	C (16) T (1) **Y** (9)	**C** (1) T (14) **A** (2) **Y** (4) **W** (5)	0 (21) 1 (5)	1
C (5 accessions)	A (4) R (1)	A (4) M (1)	C	G	T	T	A	T	C	C	T (4) K (1)	G (4) K(1)	A (4) R (1)	**T** (3) **Y** (2)	T	G	A	C	C	T	0	1
Ancestral state	A (45%) G (50%)	C (62%) A (24%)	C (78%) A (21%)	G	T	T	A	T	C	C	G (77%) C (22%)	T (56%) G (24%)	G (56%) A (43%)	C	G (95%) T (2%)	A (95%) G (4%)	A (66%) C (17%)	T (78%) C (6%)	C (28%) T (6%)	T	0 (59%) 1 (39%)	1

The frequencies of the different states within the groups are indicated in the parentheses and gene flow among the groups, suggested by shared synapomorphies, is indicated with bold letters. Ancestral states of the *H. pusillum* lineage and their ratios (in parentheses), inferred from all most parsimonious ITS trees are indicated.

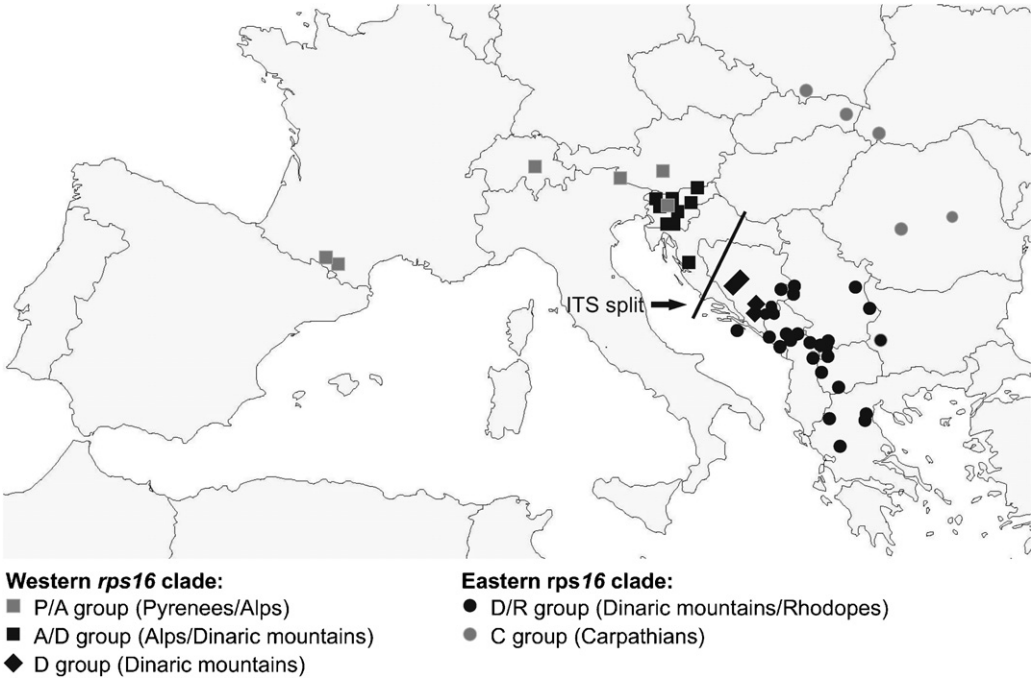

Fig. 1. Distribution of the accessions of the *H. pusillum* clade as revealed by the ITS and *rps16* data (Figs. 2 and 3). Dots correspond to the eastern *rps16* clade, squares and diamonds to the western clade. Further subgrouping is based on the ITS split network and geographic criteria: populations from the Carpathians form the C group and all remaining populations from the eastern *rps16* clade form the D/R group. The accessions from the area between the borderlines of the *rps16* clades and the ITS main split form the D group, populations from NW outskirts of the Dinaric mountains and the SE Alps form the A/D group and more divergent populations from the Alps and the Pyrenees form the P/A group.

set was analyzed with the default prior distributions, applying a JC model of evolution as proposed by the Akaike criterion implemented in MrAIC.pl version 1.4 (Nylander, 2004). The MCMC chains were run for 1,000,000 generations. Every 100th tree was saved, resulting in 10,000 trees for each data set, of which the first 2000 were discarded as a conservative characterization as the burn-in phase.

In order to display conflicts in data, we used SplitsTree4 (Huson, 1998). A pairwise (uncorrected) distance matrix generated with PAUP* (Swofford, 2002) was used to compute a NeighbourNet (Bryant and Moulton, 2004) network. The network, compared to a phylogenetic tree, potentially offers a more realistic visual presentation of possible reticulate phylogenetic patterns by depicting the incompatible signals in a net-like scheme (Huson and Bryant, 2005).

High and low elevation status was assigned to the *Heliosperma* taxa (see Section 1) and ancestral state for this character was inferred using Mesquite version 1.06 (Maddison and Maddison, 2005), with the "Trace Character Over Trees" module applying the parsimony reconstruction method over all most parsimonious ITS trees.

3. Results

3.1. Phylogenetic relationships based on the chloroplast (*rps16*) sequences

The monophyly of *Heliosperma* and its origin distinct from other *Sileneae* genera is strongly supported (100% bootstrap frequency) by the *rps16* intron sequences (Fig. 2), but the position of *Heliosperma* within *Sileneae* is not resolved. The average JC-corrected pairwise distance within *Heliosperma* is 0.0184 (SD 0.0138, $N = 1540$), whereas in the outgroup the average distance is 0.0440 (SD = 0.0146, $N = 325$). All *H. alpestre rps16* sequences are 807 bp, whereas those of *H. macranthum* are 808 bp long. The lengths of the sequences in the *H. pusillum* group vary substantially: in the western *H. pusillum* group from 818 bp (HpusCH) to 831 bp (HpudA, HpusF1, HpusF2, HmonBIH1), and in the eastern *H. pusillum* group from 781 bp (HchrGR1) to 820 bp (HtomMN1). The *rps16* matrix contains 81 taxa and 1282 characters (including 141 gap characters), of which 205 (16%) are parsimony informative. The heuristic search resulted in 8321 trees that are 570 steps long with a consistency index (CI) of 0.73 (0.61 excluding uninformative characters) and a retention index (RI) of 0.92. One of the arbitrarily chosen MP trees is shown in Fig. 2. Within *Heliosperma*, the *H. pusillum* clade has 100% bootstrap support, being a sister clade to *H. macranthum* and in turn, these form a sister clade, with 97% bootstrap frequency, to *H. alpestre*. Within the *H. pusillum* clade, there are two strongly supported groups (both with 100% bootstrap frequencies) which are geographically correlated, a western and an eastern clade (Fig. 1). Their divergence is supported by four indels, 5, 9, 7 and 3 bp long, and twelve substitutions. In the western clade two accessions from the Pyrenees (HpusF1 and F2) and one from the Alps (HpudA) group together

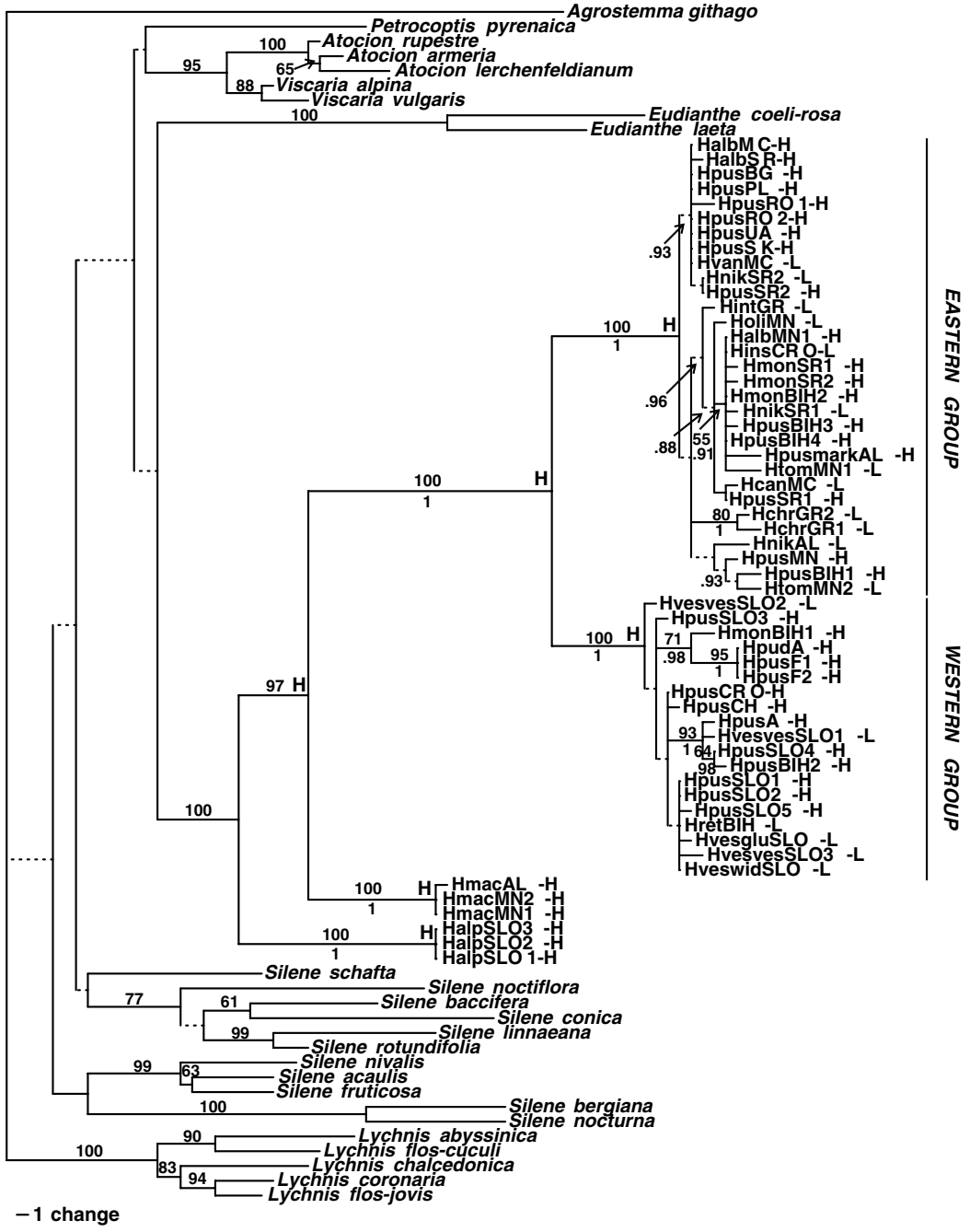

Fig. 2. Arbitrarily chosen phylogram of one of the 8321 most parsimonious trees from the analysis of the *rps16* intron sequences. Branch lengths are proportional to the inferred number of substitutions under the parsimony/DELTRAN optimization. Numbers above branches indicate parsimony bootstrap percentages over 50, and numbers below branches represent posterior probabilities obtained from bayesian analysis of *Heliosperma* taxa. Dotted branches collapse in the strict consensus tree. Species abbreviations are explained in the Table 1. According to morphological and ecological differences (Ascherson and Graebner, 1920; Neumayer, 1923; Trinajstić, 1979) high (H) and low (L) elevation status is assigned to each *Heliosperma* taxon and optimized on the branches.

with high support (four synapomorphic substitutions, 95% bootstrap support), and they share two insertions with *H. monachorum* (HmonBIH1) from Bosnia (clade with 71% bootstrap support). Another clade with strong support (93% bootstrap frequencies, three synapomorphic substitutions) groups accessions from the Alps (HpusA) and the Dinaric mountains (HvesvesSLO1, HpusSLO4, HpusBIH2). In the eastern group the accessions of *H. chromodontum* from Mt. Olympus (HchrGR1 and 2) form a clade with 80% bootstrap support. Bayesian analysis inferred similar phylogenetic relationships but there are more clades with high support indicated by posterior probabilities (depicted below branches in Fig. 2).

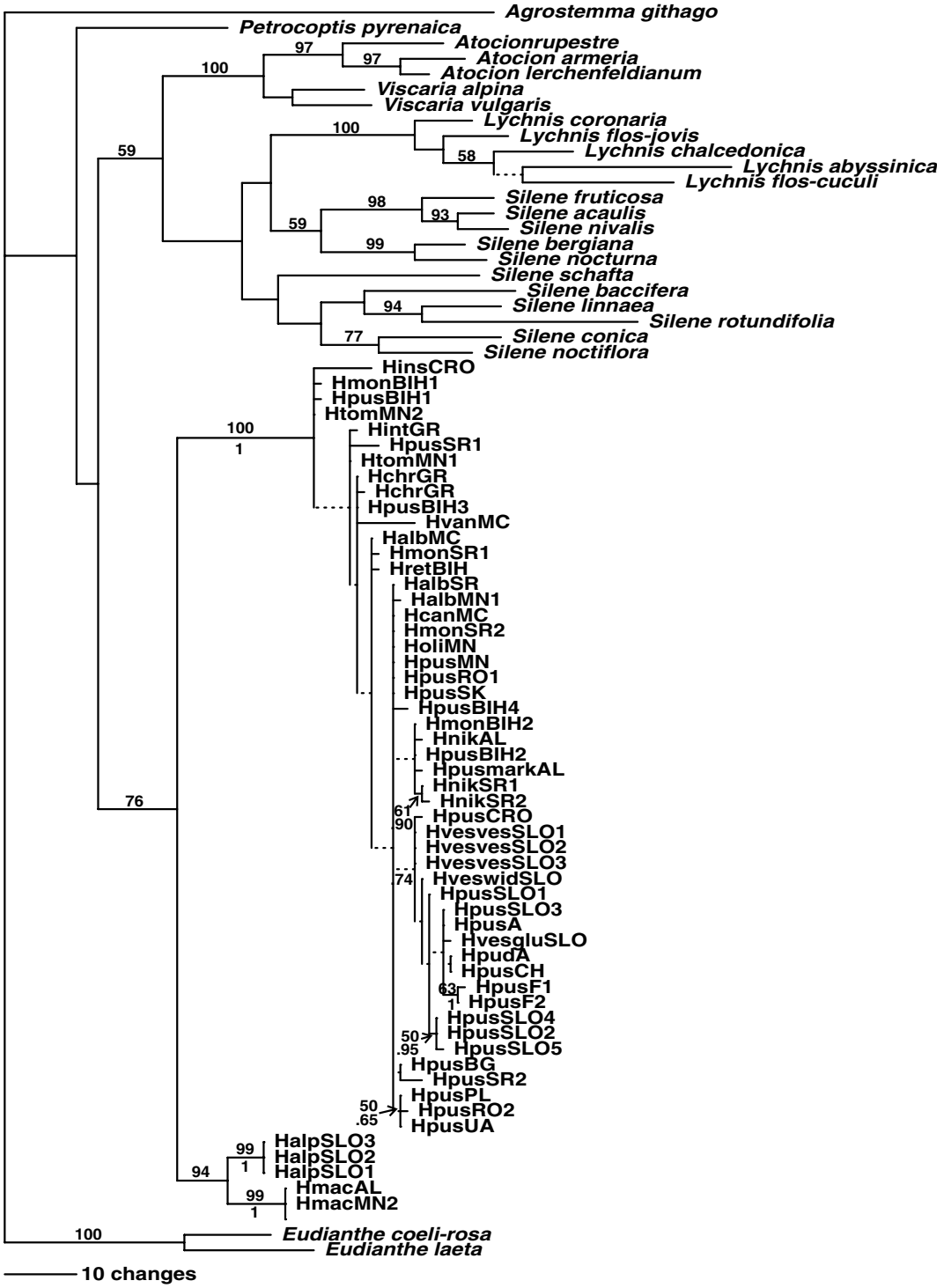

Fig. 3. Arbitrarily chosen phylogram of one of the 4073 most parsimonious trees from the analysis of the ITS sequences. Branch lengths are proportional to the inferred number of substitutions under the parsimony/DELTRAN optimization. Numbers above branches indicate parsimony bootstrap percentages over 50, and numbers below branches represent posterior probabilities obtained from bayesian analysis of *Heliosperma* taxa. Dotted branches collapse in the strict consensus tree. Species abbreviations are explained in the Table 1.

3.2. Phylogenetic relationships based on the ITS sequences

The length of the *Heliosperma* ITS1 region ranges between 243 and 245 bp, the 5.8S gene is invariably 155 bp long, and the ITS2 region ranges between 225 and 226 bp. On average, the JC-corrected pairwise distance in the ingroup is 0.0156 (SD = 0.0196, $N = 1540$), and 0.1025 (SD = 0.0337, $N = 325$). The ITS sequence matrix contains 89 taxa and 686 characters (including 41 gap characters), of which 170 (25%) are parsimony informative. The heuristic search resulted in 4073 trees of 725 steps with a consistency index (CI) of 0.49 (0.42

excluding uninformative characters) and a retention index (RI) of 0.77. One of the arbitrarily chosen MP trees is shown in Fig. 3. The position of *Heliosperma* within *Sileneae* is unresolved, but a monophyletic origin of *Heliosperma*, separated from other *Sileneae* genera, has 76% bootstrap support. There are two major sister group relationships within *Heliosperma*: the *H. pusillum* clade (with bootstrap support of 100%), including most of the taxa, is a sister clade to *H. alpestre* and *H. macranthum*, which form a clade with 94% bootstrap support. Within the *H. pusillum* clade the relationships among the taxa are poorly resolved; only four clades with bootstrap frequencies between 50% and 63% are recovered. These all comprise geographically closely allied accessions. Bayesian analysis resulted in strong support for the same clades inferred by the parsimony analysis (Fig. 3).

The NeighbourNet network from the ITS data (Fig. 4) of the *H. pusillum* group displays two geographic groups (Fig. 1). In the western group the accessions from the Pyrenees (HpusF1, HpusF2) and some from the Alps (HpusCH, HpusA, HpudA, HvesgluSLO) are the most distant, whereas the accessions from the SE Alps and the NW Dinaric mountains are less divergent. In the eastern group two splits can be observed, one formed mainly by the accessions from the Carpathians (HpusRO1 and 2, HpusUA and PL, HpusBG, HpusSR2, HcanMC), and another with three *H. nikolicii* accessions. Two taxa, *H. vandasii* and *H. insulare* (grouped with *H. monachorum*), are very divergent.

The ITS sequences of the *H. pusillum* group taxa contained several polymorphic sites (APS, Table 2). With two exceptions (characters number 166 and 195), sites polymorphic for ancestral and derived nucleotide states are either unique within a geographic region or shared between adjacent regions (Fig. 1).

4. Discussion

4.1. Monophyly of Heliosperma and phylogenetic relationships among Heliosperma taxa

The analyses of chloroplast *rps16* and nuclear ITS sequences both support that *Heliosperma* is a monophyletic group, clearly separated from the core of *Silene*, but the position of *Heliosperma* within *Sileneae* remains unclear. However, our results show that the position of *Heliosperma* as a sister to *Viscaria vulgaris* as presented by Oxelman and Lidén (1995), Oxelman et al. (1997) and Oxelman et al. (2001) is erroneous. Probably, the putative *H. pusillum* sequence used in their analyses was based on a *Viscaria alpina* seedling which had been mixed up with *Heliosperma* seedlings in the greenhouse.

Our results clearly show that Neumayer's (1923, 1924) *Heliosperma* species delimitations are in agreement with a phylogenetic species concept. There are two species clearly separated from the *H. pusillum* group (consisting of all other taxa): *H. alpestre* from the Alps and *H. macranthum* from the mountains in Montenegro and Albania, which are

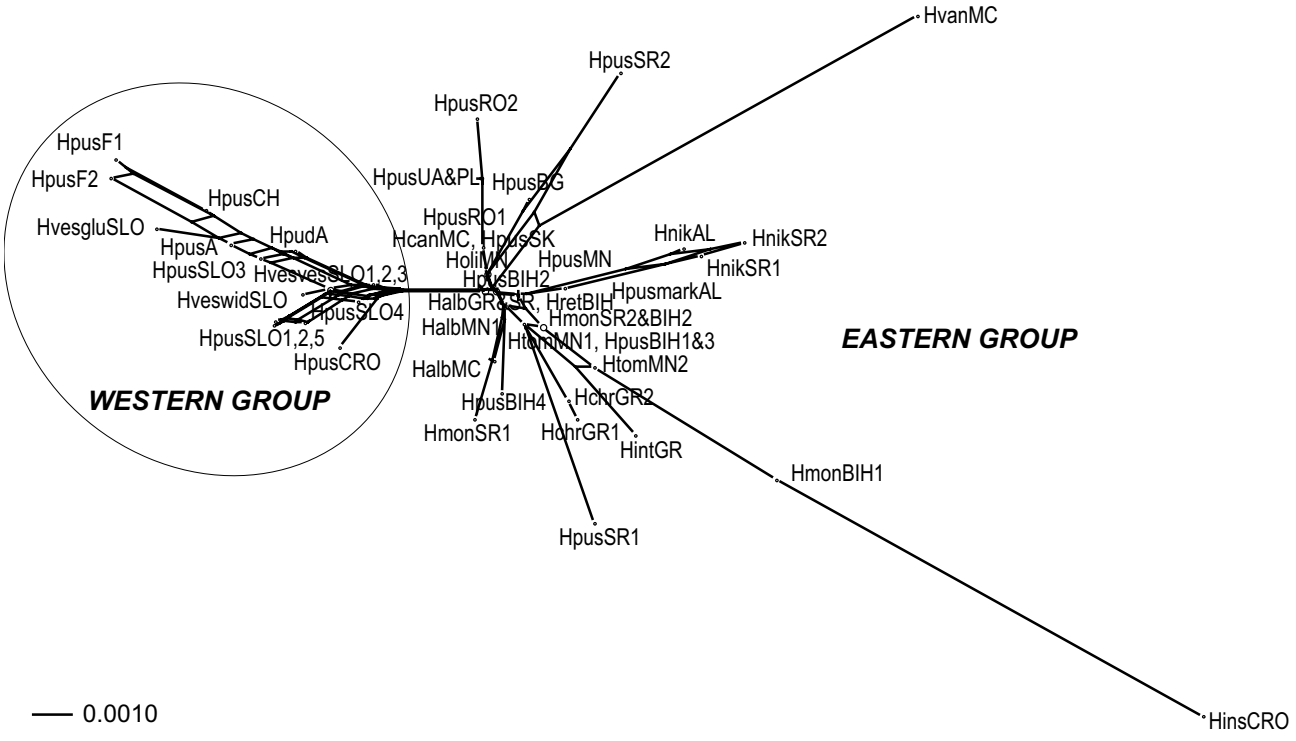

Fig. 4. Splits graph for ITS sequences from 51 accessions of the *H. pusillum* group. Branch lengths were estimated using the standard implementation of split decomposition (SplitsTree4.0 beta 06). The western and the eastern groups are indicated, corresponding to the clades revealed by the *rps16* sequences within the *H. pusillum* group (Fig. 2, see discussion for further explanation). Species abbreviations are explained in the Table 1.

both morphologically clearly differentiated. The hypothesis of Niketić and Stevanović (in press) that *H. oliverae* might be a link between *H. macranthum* and other *Heliosperma* endemics, included within the *H. pusillum* clade, is not supported by ITS and *rps16* phylogenies: *H. oliverae* is positioned within the *H. pusillum* clade.

The relationships among *H. alpestre*, *H. macranthum* and the *H. pusillum* lineages are different in the ITS and *rps16* phylogenies. *H. alpestre* and *H. macranthum* together form a sister clade to the *H. pusillum* clade in the phylogenetic tree inferred from the ITS sequences, whereas the *rps16* data suggest a sister group relationship between *H. macranthum* and the *H. pusillum* lineage. The incongruence could be caused by either lineage sorting or by ancient hybridization (see e.g. Rieseberg and Wendel, 1993; Rieseberg et al., 1996; Okuyama et al., 2005). There are no APS in the *H. macranthum*/*H. alpestre* ITS sequences, whereas they are abundant in the *H. pusillum* clade (see below). A few sites in the *H. pusillum* group are APS with either *H. alpestre* or *H. macranthum*. If ancient hybridization is responsible for the conflicting relationships among the three major groups in *Heliosperma*, concerted evolution must have had sufficient time to homogenize the ITS sequences to the paternal type (assuming maternal inheritance of plastids).

4.2. Phylogeographic patterns within the H. pusillum group

The ITS and *rps16* sequences both strongly support monophyly of the *H. pusillum* group. Most of the relationships within the group are poorly resolved, but there are two strongly supported clades in the *rps16* intron tree, which are not found in the ITS tree. However, the ITS NeighbourNet network displays two groups with similar content as the *rps16* clades (Figs. 1 and 3). The two groups do not reflect morphological or taxonomical boundaries, but are strongly geographically correlated (the western and eastern groups in Fig. 1). The borderline between the *rps16* clades is the border region between Serbia and Bosnia/Herzegovina, whereas the borderline between the ITS geographic groups is about 150 km to the northwest. Since we sequenced only one specimen per population, it is possible that chloroplast haplotype groups are present throughout the distribution of *Heliosperma*, but the relatively large sample size strongly rejects the idea that the clear geographical structure would be due to sampling error. Sharing of the same chloroplast types among different species from the same geographical areas has been observed in other plant groups (e.g. Rieseberg et al., 1996; Wolf et al., 1997; Gutierrez Larena et al., 2002; McKinnon et al., 2001; Gardner et al., 2004; Okuyama et al., 2005). Introgression followed by relatively rapid fixation (due to the small population size of plastids) of an introgressed haplotype (chloroplast capture) has been invoked as an explanation in several of these studies. However, chloroplast capture would likely result in a more mosaic geographic pattern than observed in the case of *Heliosperma*. The fact that the NeighborNet analysis of the ITS data indicates a major bipartitioning that is fairly congruent with the *rps16* data also speaks against chloroplast capture as a sensible explanation to the observed pattern. Alternative explanations are ancient divergence and isolation of the two *H. pusillum* lineages, followed by more recent hybridization events, or incomplete lineage sorting of ancestors polymorphic for the ITS region together with incomplete concerted evolution. It is well known that hybrids between distantly related taxa can seriously distort an otherwise hierarchical tree structure (e.g. McDade, 1992), and incomplete concerted evolution between distantly related rDNA repeats could cause similar problems (Alvarez and Wendel, 2003). We favor hybridization as the most likely explanation for two reasons. First, concerted evolution of rDNA repeats is often considered to be a rapid process (Alvarez and Wendel, 2003). If this is the case, the high incidence of additive polymorphic sites (APS) in the ITS sequences within the *H. pusillum* group indicates extensive recent processes that are likely to be much younger than the split between the cpDNA groups. Second, the apparent similar substitution rates of the ITS and *rps16* sequences in *Heliosperma* is at odds to the general expectation that ITS substitution rates are higher than those of non-coding cpDNA regions (e.g. Small et al., 2004). Hybridization between previously isolated ITS lineages could potentially tend to homogenize them in a fashion similar to what has been documented in several other groups of hybrid origin (e.g. Zhang and Sang, 1999; Small et al., 2004). If the extinction of ribotypes in different hybrids is non-random, then the reduced sequence diversity would appear as a reduced substitution rate. This is consistent with what we observe in *Heliosperma* and the rest of *Sileneae*, where the sequence divergence of ITS is more than twice that of the *rps16* intron. Admittedly, the ITS sequences do not lend themselves to an easy interpretation, and it is possible that a combination of different processes is responsible for the non-tree-like pattern observed. Future studies using low copy-number nuclear loci and intrapopulation variation should be able to test the hybridization hypothesis. For example, in a simple rooted three-taxon case using several low-copy loci, all three topologies should be recovered if lineage sorting is the cause of the conflicting signal, whereas a single hybridization event would imply that only two conflicting topologies should be present.

The incongruent topologies of the *rps16* and ITS trees, as well as the continuous morphological variability of *H. pusillum* across its distribution, without any signs of morphological divergence between the eastern and western group populations (Ascherson and Graebner, 1920; Neumayer, 1923; Trinajstić, 1979; pers. observ.), is consistent with the idea that hybridization and gene flow could be responsible for at least some of the polymorphic sequence patterns (Table 2). Geographic patterning of nuclear ITS and chloroplast sequences has also been observed in

Clausia aprica (Franzke et al., 2004), but there the ITS APS were found exclusively in the contact area of the previously isolated lineages (as indicated by the cpDNA data), whereas the geographically more distant areas retained the pattern revealed by the chloroplast sequences. In the *H. pusillum* group, APS are found over the entire distribution, but are less abundant in the geographical extremes, that is, the Pyrenees, western Alps, and Carpathians (groups P/A and C in Table 2).

At almost all APS positions in the ITS matrix (Table 2) one of the bases was present in accessions from all geographic groups (Fig. 1), whereas the other base, either fixed or as an APS, was present only in accessions of some geographic groups. The common base is in several cases the same as the inferred ancestral state. In these cases, the geographical distribution of novel states can be interpreted as indication of gene flow that occurred after the separation of the cpDNA groups. For example, the APS's at nucleotide positions 180, 187, and 582, and indel at nucleotide position 681 indicate gene flow between the groups P/A and A/D (Table 2). A detailed analysis of the distribution of less common bases (either indicated by APS, or fixed) at different nucleotide positions among different geographic groups indicates gene flow along the Dinaric mountains, i.e. between the eastern and the western *rps16* clades.

A biogeographic alliance between the Dinaric mountains and the SE Alps has long been known (Turrill, 1929). At the contact zone between the Alps and Dinaric mountains in Slovenia one can observe a mixture of Alpine and Dinaric elements (Wraber, 1990). Alliance between the Pyrenees/Alps and the southern Dinaric mountains (groups P/A-(D)-D/R), indicated by shared bases at two nucleotide positions is surprising due to a great geographic distance between the two areas. Homoplasy cannot be ruled out, but it is difficult to assess whether the sharing is more likely to be due to homoplasy, hybridization, or shared ancestral polymorphism. Sharing of apomorphic bases between the populations in the Dinaric mountains/Rhodopes and the Carpathians, as well as their grouping in the same *rps16* clade, is consistent with the idea that the Carpathian populations may have originated from the eastern margins of the Dinaric mountains. The Iron Gates along the Danube in Serbia/Romania have been considered as a possible migration route between the Carpathians and the Dinaric mountains (Reed et al., 2004). There are no indications for gene flow between the Carpathians and the Alps and/or Pyrenees. No gene flow is indicated between the Alps and the Tatra mountains, even if a close relationship of eastern Alpine and Tatra/Carpathian populations has previously been reported (e.g. Kropf et al., 2003).

Without any phylogenetic dating available for the *Sileneae* yet, it is premature to draw any conclusions about the timing of putative hybridization events, but climatic oscillations during the Pleistocene might have caused range fragmentations of *Heliosperma* and subsequent secondary contacts following range expansions. The divergence between the two major chloroplast lineages of *H. pusillum* is deep. Applying a molecular clock and a Jukes-Cantor model of sequence evolution to the tree in Fig. 1 gives a conservative estimate of at least one million years since the divergence between the two groups, if a fast calibrated rate of evolution of non-coding chloroplast sequences is applied (8.24×10^{-9} substitutions per site per year, cf. Richardson et al., 2001). More detailed dating of the *Sileneae* phylogeny is being prepared (Eggens et al., in prep.), but a pre-Pleistocene divergence of the two groups appears reasonable.

4.3. The H. pusillum group—morphology and ecology vs. genes

The differentiation of *H. pusillum* sensu lato into two morphologically and ecologically defined groups, i.e. the low and the high elevation groups is not supported by the molecular data (Figs. 2 and 3). As revealed by the *rps16* phylogenetic tree, if high/low elevation forms are considered as character states, high elevation is parsimoniously optimized as the plesiomorphic state (Fig. 2), since this is the state for *H. alpestre* and *H. macranthum*. This is consistent with the ideas of Neumayer (1923). The low elevation taxa do not form a monophyletic group, but rather nest among the high elevation taxa (Figs. 1 and 2), indicating that the morphological character combination found in the low elevation taxa has multiple origins. Neumayer (1923) proposed a post Pleistocene origin and diversification of low elevation *H. pusillum* group taxa, but the climatic oscillations during the Pleistocene and consequent migrations of the biota, as well as fragmentations of distribution ranges are perhaps a better explanation for such a pattern, as suggested also by Niketić and Stevanović (in press). Altitudinal differentiation during the Pleistocene might have been possible, since the snow line at the Balkan Peninsula was lower compared to the other Southern European mountains (Turrill, 1929). Glacial-induced altitudinal migrations and subsequent differentiations have been documented in *Armeria* (Gutierrez Larena et al., 2002). Low elevation *H. pusillum* taxa have found their present refugia in gorges, growing in rock crevices, where competition with other plants is low. Here they adapted to the specific and similar environmental conditions which may explain their overall morphological similarity.

Turrill (1929) noted that altitudinal migration of plants and their subsequent morphological differentiation, caused by the modifying environmental factors, has had an important influence on how taxonomists have classified the Balkan flora. If a proposed altitudinal (ecological) differentiation of the *H. pusillum* group taxa is an explanation for the observed morphological differences, it is expected that low elevation populations are more closely related to the high elevation populations in the same small-scale geographic range (e.g. the same river basin) than to the other low elevation populations (taxa). Our

sampling at a smaller geographic scale was not extensive enough to be able to test this hypothesis, with the exception of two low-high elevation assemblies (HalbMC-HalbMC-HnikSR1-HnikSR2 and HpusBIH1-HpusBIH3), but they did not show any increased genetic alliance compared to other accessions. However, recurrent gene flow could have blurred such patterns.

Additional studies are needed to better understand the patterns and processes in the evolutionary history of *Heliosperma*. In order to disentangle its reticulate phylogenetic history, there is a strong need for alternative regions to chloroplast DNA and ITS. More extensive geographic sampling as well as sequencing additional nuclear genes could help addressing the apparently complex phylogenetic history of *Heliosperma*. Moreover, we hope that this research will foster additional studies on the phylogenetic and phylogeographic patterns of other Balkan plants.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ympev.2006.11.003.

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