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Allopolyploid origin of the Balkan endemic *Ranunculus wettsteinii* (Ranunculaceae) inferred from nuclear and plastid DNA sequences

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Abstract The Balkan Peninsula, characterized by high rates of endemism, is recognised as one of the most diverse and species-rich areas of Europe. However, little is known about the origin of Balkan endemics. The present study addresses the phylogenetic position of the Balkan endemic *Ranunculus wettsteinii*, as well as its taxonomic status and relationship with the widespread *R. parnassiifolius*, based on nuclear DNA (internal transcribed spacer, ITS) and plastid regions (*rpl32-trnL*, *rps16-trnQ*, *trnK-matK* and *ycf6-psbM*). Maximum parsimony and Bayesian inference analyses revealed a well-supported clade formed by accessions of *R. wettsteinii*. Furthermore, our phylogenetic and network analyses supported previous hypotheses of a likely allopolyploid origin for *R. wettsteinii* between *R. montenegrinus* and *R. parnassiifolius*, with the latter as the maternal parent.

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Introduction

The Balkan Peninsula shows one of the highest degrees of species richness and endemism of Europe, and is recognised widely as a centre of biodiversity in Europe (Gaston and David 1994). However, detailed phylogeographic and molecular systematic studies of the Balkan biota are rare because sampling has been restricted until recently, due to conflicts and wars in the area (however see e.g. Park et al. 2006; Frajman and Oxelman 2007; Kučera et al. 2008; Magyari et al. 2008; Slovák et al. 2009; Lo Presti et al. 2010; Mereďa et al. 2011; Surina et al. 2011), so the level of knowledge is poorer than in other areas (but see Bittkau and Comes 2005; Albach et al. 2009; Surina et al. 2011).

The genus *Ranunculus* L. (buttercups) has a cosmopolitan distribution and comprises about 600 herbaceous species (Tamura 1995; Hörandl et al. 2005; Emadzade et al. 2010; Hörandl and Emadzade 2012). It is the largest genus of Ranunculaceae and one of the 50 largest genera of angiosperms (Frodin 2004). Recent molecular studies (Cires et al. 2009, 2010, 2012; Cires and Fernández Prieto 2012) present a new evolutionary scenario for the European group *Ranunculus parnassiifolius*. However, it is worth stressing that there is still considerable taxonomic confusion regarding the isolated population of *Ranunculus wettsteinii* Dörfler, known only from the type locality on Mt. Korab/Maja e Korabit (Albania-Macedonia), including its phylogenetic position. Some authors (e.g. Baltisberger 1992) consider it a separate species, while others (e.g. Tutin and Akeroyd 1993) view it as a subspecies of *R. parnassiifolius*.

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In 1918 Ignaz Dörfler discovered *R. wettsteinii* in screes of Cüseli (= Kula Ziberit, Korab, Albania-Macedonia). He sampled a large number of plants (under the sampling number 760) and distributed duplicates to many European herbaria (BM, BP, BRNU, C, E, G, GB, K, LD, LJU, M, S, WU, Z, ZT; see http://sweetgum.nybg.org/ih/). The same area was later visited by Vojtek Lindtner in 1936, who also collected plants of *R. wettsteinii* and distributed duplicates to E, GB, GZU, K, LD, LJU. According to Dörfler (1918) and Hayek (1924), *R. wettsteinii* differs morphologically from *R. parnassiifolius* by the presence of broadly lobed leaves, multifloral stems and smaller flowers.

The northern area of Kula Ziberit consists of siliceous screes where *R. wettsteinii* occurs on acidic substrate (approximately pH 5.0 at the surface, pH 4.5 at 15 cm depth; see Baltisberger 1992). Morphologically only 10 % of the individuals could be determined as *R. wettsteinii*, whereas 90 % resemble *R. parnassiifolius* (Baltisberger 1992). The same chromosome number ($2n = 4x = 32$) was determined for five plants of each morphological type (Baltisberger 1992). On the other hand, chromosome counts and recent genome size studies using flow cytometry, revealed two predominant DNA ploidy levels (diploid and tetraploid) for *R. parnassiifolius* throughout Europe (Cires et al. 2009, 2010).

It is interesting that *R. wettsteinii* morphologically resembles the triploid ($2n = 3x = 24$) hybrid *R.* × *digeneus* Kerner ex Huber (=*R. parnassiifolius* x *R. seguieri*) nothosubsp. *digeneus* described by Huber (1988) from the Dolomites (Italian Alps); however, the pentaploid ($2n = 5x = 40$) nothosubsp. *latemarensis* Huber is morphologically different. Based on this evidence, Baltisberger (1992) postulated a hybrid (allopolyploid) origin for *R. wettsteinii* in which the tetraploid *R. montenegrinus* (=*R. seguieri* subsp. *montenegrinus* (Halácsy) Tutin) would be involved.

Considering the unclear origin as well as the different taxonomic treatments of *R. wettsteinii*, the goals of the present study were to: (1) determine its phylogenetic position within the genus *Ranunculus*, (2) explore its hypothetical hybrid origin using molecular data (ITS, *rpl32-trnL*, *rps16-trnQ*, *trnK-matK, ycf6-psbM*), and (3) propose a taxonomic status of *R. wettsteinii* based on phylogenetic relationships within the polyploid complex *R. parnassiifolius*.

Materials and methods

Plant material and sampling

We extended our previous sampling (Cires et al. 2012) to include plants of the group *Ranunculus parnassiifolius* from the type locality of *R. wettsteinii* (including both morphological types—the typical *R. wettsteinii* and plants resembling *R. parnassiifolius*), as well as putatively closely related species *R. montenegrinus* and *R. seguieri* based on previous studies (Baltisberger 1992). GenBank accession numbers are provided in Appendix.

DNA extraction, amplification and sequencing

DNA extraction was conducted as described in Cires et al. (2012). The PCR reaction mix of 25 μL contained 25 ng template DNA, 2.5 μL 10x PCR buffer, 2.5 mM $MgCl_2$, 0.2 mM dNTPs, 1 U *Taq* DNA polymerase (Invitrogen, Carlsbad, CA) and 0.08 μM of each primer [17SE-26SE of Sun et al. (1994) for nuclear ITS; *rpl32F-trnL*^{UAG} of Shaw et al. (2007); *rps16x1-trnQr* of Dumolin-Lapègue et al. (1997) and Shaw et al. (2007); *trnK3914F-matK1470R* of Johnson and Soltis (1994); *ycf6-psbM* of Shaw et al. (2005) for plastid sequences]. PCR conditions were 5 min at 94 °C followed by 40 cycles of 1 min at 94 °C, 1 min at 56 °C, 1 min at 72 °C; plus a final extension of 10 min at 72 °C. To remove primers and unincorporated dNTPs, PCR products were treated with ExoSAP-IT (USB Corporation, Cleveland, OH) following the manufacturers' protocols. Cleaned products were sequenced directly using dye terminators (Big Dye Terminator v. 2.0, Applied Biosystems, Little Chalfont, UK). Both strands were sequenced to check the reliability of detected differences. All chromatograms were examined visually to correct possible misinterpretations of the computational routine. Sequence data was assembled and edited using ClustalW v.1.83 algorithm implemented in Geneious Pro 5.3 (Biomatters, Auckland, New Zealand). IUPAC (International Union of Pure and Applied Chemistry) symbols were used to represent nucleotide ambiguities.

Phylogenetic and network analyses

Phylogenetic reconstruction of both ITS and concatenated plastid datasets, respectively, was undertaken using maximum parsimony (MP) and Bayesian inference (BI). MP analysis was conducted by heuristic search using MEGA 5 (Tamura et al. 2011). The most parsimonious tree was obtained using the Tree-Bisection-Regrafting (TBR) algorithm (Nei and Kumar 2000) with search level 5 in which the initial trees were obtained by the random addition of sequences (ten replicates) and gaps treated as missing data. The robustness of nodes was inferred from a bootstrap analysis (BP) of 3,000 replicates. Standard parameters such as consistency index and retention index (CI and RI, respectively) were also calculated. Different partitions of the five datasets (ITS, *rpl32-trnL*, *rps16-trnQ*, *trnK-matK* and *ycf6-psbM*) were tested separately using MrModeltest v.2.3 (Nylander 2004) to determine the best evolutionary evolution model that described the present data. The best fitting models of evolution were SYM+I for ITS, HKY+I for *rpl32-trnL*, HKY+G for *trnQ-rps16*, F81+I for *trnK-matK* and F81+I for *ycf6-psbM*, using the Akaike Information Criterion (AIC). Bayesian Inference (BI) analysis

Table 1 Sequence characteristics of *Ranunculus* obtained from the analysis of ITS, *rpl32-trnL*, *rps16-trnQ*, *trnK-matK*, *ycf6-psbM* sequences

	ITS	*rpl32-trnL*	*trnQ-rps16*	*trnK-matK*	*ycf6-psbM*
Substitution model	SYM+I	HKY+I	HKY+G	F81+I	F81+I
Length range (bp)	611-614	909-920	1003-1029	1366-1384	1164-1257
Aligned length (bp)	615	949	1066	1412	1274
Constant characters	574	850	925	1320	1109
Polymorphic sites	41	99	141	92	165
Number of indels (%)	108 (0.6 %)	1058 (3.7 %)	1702 (5.3 %)	1090 (2.6 %)	2096 (5.5 %)
Mean G + C content (%)	55.0 %	28.4 %	28.4 %	30.2 %	31.0 %

was conducted using MrBayes v.3.2.0 (Ronquist et al. 2012), with each substitution model fitted to its specific DNA partition. Four Markov chains were run simultaneously for 10 million generations, and they were sampled every 100 generations. Chain convergence was assessed with Tracer v1.5 (Rambaut and Drummond 2007), and a 50 % majority rule consensus tree with Bayesian posterior probabilities (PP) of clades was calculated to obtain the Bayesian estimate of phylogeny, after removing the first 20 % generations as burn-in. Homogeneity between the plastid and nuclear datasets was tested using the incongruence length difference (ILD) test (Farris et al. 1995) as implemented in PAUP* v.4.0b10 (Swofford 2002). The ILD test was conducted with 1,000 replicates, TBR branching swapping, and keeping no more than 100 trees per random addition replicate. BI support values (posterior probability) are indicated on tree branches as percentages. For the network analysis, we used the NeighborNet algorithm implemented in SplitsTree v.4.11 (Huson and Bryant 2006) to construct an unrooted network, applying uncorrected distances (also known as p-distances or Hamming distances). Bootstrap support for internal splits was calculated with 1,000 replicates, and non tree-like splits graphs indicate contradictory support for relationships. Fit values ranging from 0 to 100 % indicate how well the information contained in the data is represented in the graph.

Results

The characteristics of the nuclear (ITS) and plastid (*rpl32-trnL*, *rps16-trnQ*, *trnK-matK*, *ycf6-psbM*) sequences used here from the genus *Ranunculus* are summarised in Table 1. Within *Ranunculus wettsteinii*, the length of the aligned ITS sequences was 612 base pairs (bp), with a mean GC content of 55.1 %. In the case of the combined plastid regions, the sequences varied in length from 4,459 to 4,471 bp, with a mean GC content of 29.5 %. Variable sites of the datasets are summarised in Table 2, which includes polymorphic sites of ITS sequences between *R. wettsteinii* and the different subspecies of *R. parnassiifolius*.

MP analyses performed on the ITS data set (31 accessions, 615 aligned characters) produced six most parsimonious trees (CI=0.72; RI=0.93). Tree topologies from BI analyses based on ITS were congruent with the consensus tree obtained using MP analysis (Fig. 1). Both phylogenetic trees depicted a clade comprising all the accessions of *R. amplexicaulis*, *R. cabrerensis s.l.* and *R. parnassiifolius s.l.* (98 % BP; 96 PP). On the other hand, all the accessions of *R. wettsteinii* formed an independent and well-supported subclade (72 % BP; 96 PP) including the morphologically resembling *R. montenegrinus*.

The phylogenetic trees based on 31 accessions from plastid dataset included 4,834 characters (1,030 from *rpl32-trnL*; 1,078 from *rps16-trnQ*; 1,416 from *trnK-matK*; 1,310 from *ycf6-psbM*), of which 497 were variable and informative. The MP analysis produced two most parsimonious trees (CI=0.76; RI=0.93). The BI analysis of the plastid data displayed similar resolution and topology as the MP analysis (Fig. 2). The phylogenetic trees revealed that all accessions of *R. wettsteinii* form a well-supported monophyletic lineage (71 % BP; 99 PP), very close to the *R. parnassiifolius s.l.* and *R. amplexicaulis*.

According to the partition homogeneity test, the nuclear and plastid datasets were significantly incongruent (P=0.01). However, several studies have shown some concerns about the reliability of ILD (Dolphin et al. 2000). For instance, significant ILD P-values may not indicate genealogical conflicts but other issues such as different evolutionary rates between the data partitions (e.g. Hipp et al. 2004). Despite the inconsistency mentioned, we have combined plastid and ITS data to increase the phylogenetic resolution, following the same approach as Paun et al. (2005) in other studies of the genus *Ranunculus*. In Fig. 3, we show NeighborNet splits graphs for nuclear ITS data (3a), for plastid data (3b) and for concatenated ITS and plastid regions (3c). The allopoplyploid *R. wettsteinii* is visualised as intermediate between two distinct lineages (*R. montenegrinus* and *R. parnassiifolius*). Considering the combined analysis of ITS and plastid data, all accessions from Korab (including typical *R. wettsteinii* as well as the individuals resembling *R. parnassiifolius*; for details regarding each accession see Appendix) formed a highly supported monophyletic group (93.7 % BP). *Ranunculus*

Table 2 Polymorphic sites in the datasets of ITS and plastid (*rpl32-trnL*, *rps16-trnQ*, *trnK-matK*, *ycf6-psbM*) sequences for *Ranunculus wettsteinii* and subspecies of *R. parnassiifolius*. Superscripts for each taxa correspond to accessions presented in the Appendix

Taxon – Ploidy Level	ITS													*rpl32-trnL*										*rps16-trnQ*										
	012	060	074	099	135	143	153	214	221	435	591	598	599	001	020	021	032	781	782	783	784	785	903	349	436	437	474	503	511	528	541	560	565	575
R. wettsteinii[1] – 4x	C	T	C	T	C	C	A	C	A	A	A	A	G	C	-	-	-	T	T	C	T	T	T	T	C	-	T	T	T	A	A	-	T	A
R. wettsteinii[3] – 4x	C	T	C	G	C	C	A	C	A	A	A	A	G	C	-	-	A	T	T	C	T	T	-	T	C	A	A	-	-	-	-	C	T	-
R. p. subsp. *favargeri*[3] – 2x	-	C	T	T	T	T	C	T	T	G	G	C	A	A	A	T	A	-	-	-	-	-	T	C	-	A	A	-	-	-	-	C	-	-
R. p. subsp. *favargeri*[1] – 4x	-	C	T	T	T	T	C	T	T	G	G	C	A	A	A	T	A	-	-	-	-	-	T	C	-	A	A	-	-	-	-	C	-	-
R. p. subsp. *heterocarpus*[3] – 4x	-	C	T	T	T	T	C	T	T	G	G	C	G	A	A	T	A	T	T	C	T	T	T	C	-	A	A	-	-	-	-	C	-	-
R. p. subsp. *parnassiifolius*[1] – 2x	-	C	T	T	T	T	C	T	T	G	G	C	G	A	A	T	A	T	T	C	T	T	T	C	-	A	A	-	-	-	-	C	-	-
R. p. subsp. *parnassiifolius*[2] – 4x	-	C	T	T	T	T	C	T	T	G	G	C	G	A	A	T	A	T	T	C	T	T	T	T	-	A	A	-	-	-	-	C	-	-

Taxon – Ploidy Level	*trnK-matK*	*ycf6-psbM*							
	580	086	442	548	577	604	608	623	627	1076
R. wettsteinii[1] – 4x	-	A	A	T	-	T	-	A	-	-
R. wettsteinii[3] – 4x	G	A	-	-	A	-	-	-	-	-
R. p. subsp. *favargeri*[3] – 2x	-	A	-	-	A	-	-	-	-	-
R. p. subsp. *favargeri*[1] – 4x	-	-	-	-	A	-	-	-	-	-
R. p. subsp. *heterocarpus*[3] – 4x	-	A	-	-	A	-	-	-	-	-
R. p. subsp. *parnassiifolius*[1] – 2x	-	A	-	-	A	-	G	-	G	T
R. p. subsp. *parnassiifolius*[2] – 4x	-	A	-	-	A	-	-	-	-	-

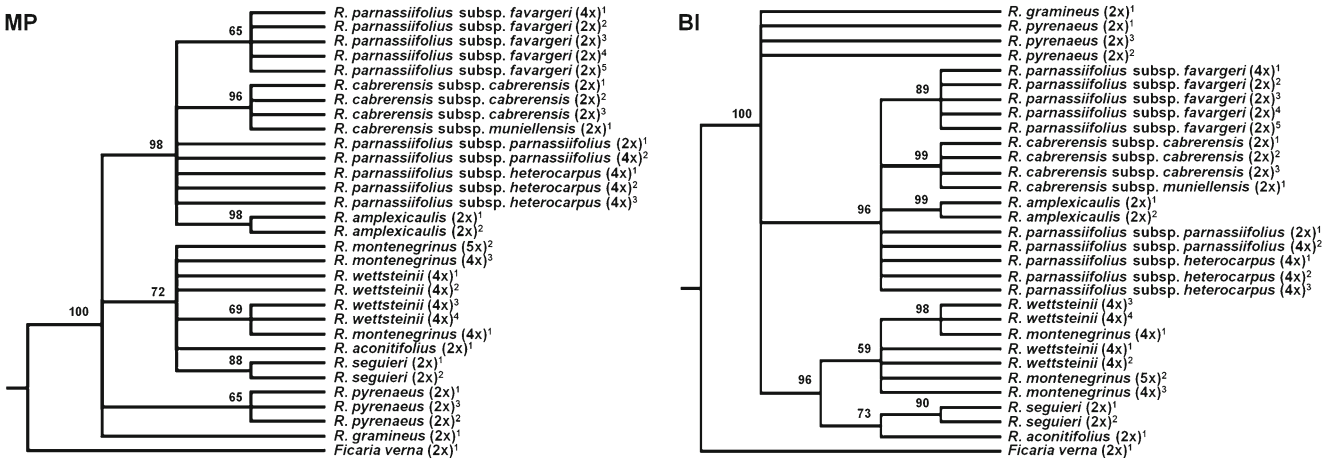


Fig. 1 Phylogenetic tree for *Ranunculus* species based on internal transcribed spacer (ITS) sequences. **a** Consensus tree inferred from the six most parsimonious trees (CI=0.72; RI=0.93). Numbers above branches show bootstrap values (3,000 replicates). **b** Majority-rule consensus of 150,002 trees derived from Bayesian inference (BI) analysis. BI support values (posterior probability) are indicated on tree branches as percentages. *Ficaria verna* (=*R. ficaria*) was used as the outgroup to root each tree

wettsteinii was sister to the accessions of the three subspecies of *R. parnassiifolius*. Finally, a relatively distant relationship was found between *R. wettsteinii* and the two putative parental species *R. montenegrinus* and *R. seguieri*.

Discussion

The Balkan Peninsula represents one of the most significant biodiversity hotspots in Europe (Gaston and David 1994; Kryštufek and Reed 2004), and the role of the region as the principal area of persistence of many European temperate taxa throughout both glacial and interglacial stages of the Quaternary has been often emphasised (Bennett et al. 1991; Tzedakis et al. 2002). Like other southern European peninsulas (i.e. Iberian and Apennine), the Balkan peninsula acted as major glacial refugium for many European species of plants (and animals) during the climatic extremes of the Pleistocene ice ages (Ibrahim et al. 1996; Taberlet et al. 1998; Hewitt 2000; Ehlers and Gibbard 2003). As an example of this floristic richness, the present study has determined the phylogenetic position of the Balkan endemic *Ranunculus wettsteinii* and its relationship with the widespread *R. parnassiifolius.*

Recent molecular studies (Cires et al. 2009, 2010, 2012; Cires and Fernández Prieto 2012) did not recognise the group *Ranunculus parnassiifolius* as formerly described, but proposed a new evolutionary scenario for this group, in which the diploid *Ranunculus cabrerensis* should be considered an independent species of the diploid-polyploid complex *R. parnassiifolius*. Within this new evolutionary scenario, *R.*

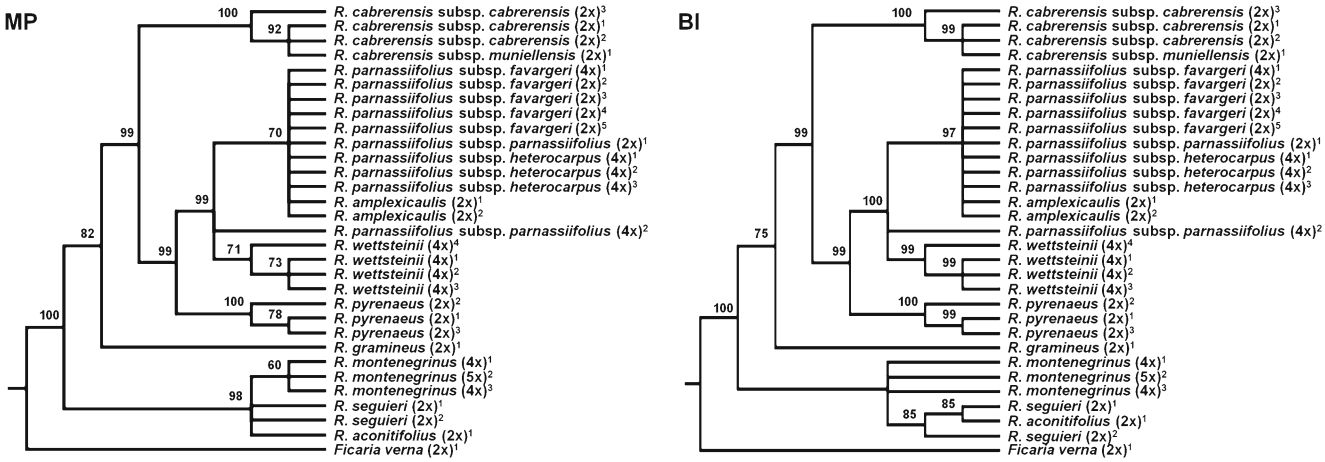


Fig. 2 Phylogenetic tree for *Ranunculus* species based on plastid regions (*rpl32-trnL*, *rps16-trnQ*, *trnK-matK*, *ycf6-psbM*). **a** Consensus tree inferred from the two most parsimonious trees (CI=0.76; RI=0.93). Numbers above branches show bootstrap values (3,000 replicates). **b** Majority-rule consensus of 148,502 trees derived from Bayesian inference (BI) analysis. BI support values (posterior probability) are indicated on tree branches as percentages. *Ficaria verna* (=*R. ficaria*) was used as the outgroup to root each tree

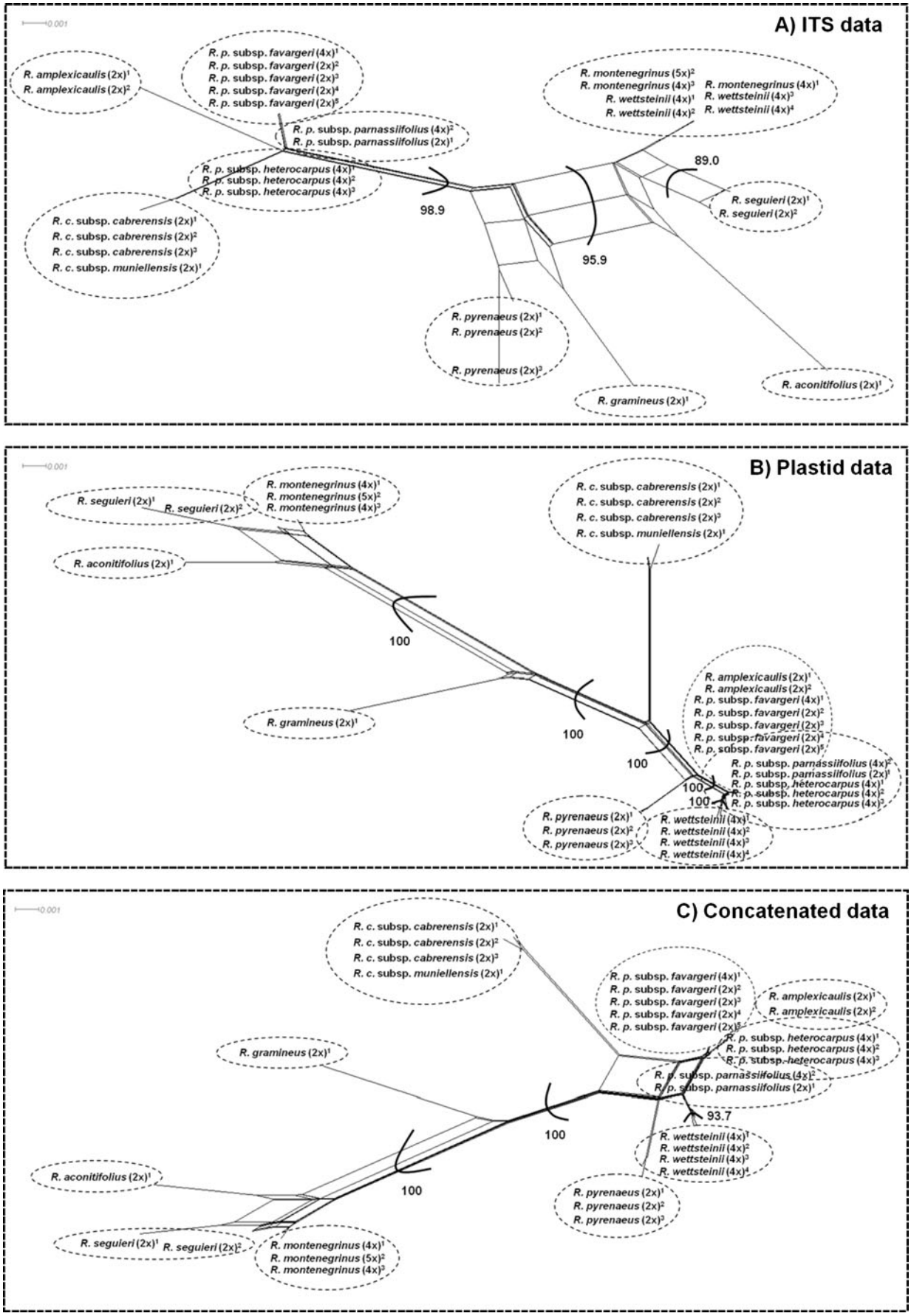
parnassiifolius subsp. *parnassiifolius* includes diploids and tetraploids from the Pyrenees; *R. parnassiifolius* subsp. *heterocarpus* are apomict tetraploids plants from the Alps, and finally, *R. parnassiifolius* subsp. *favargeri* is formed by diploids and tetraploids from the Cantabrian Mountains. In the case of *R. amplexicaulis*, this species is positioned in the same clade as *R. parnassiifolius*, as demonstrated in previous studies (Cires et al. 2012). A considerable taxonomic confusion has always surrounded the isolated population of *R. wettsteinii* from Mt. Korab. Based on our results, the recognition of *R. wettsteinii* at the species level as a genetically and morphologically separate lineage is supported (see Fig. 3, Table 2). Although this species is characterised by the presence of broadly lobed leaves, some individuals are lacking this key character and morphologically resemble *R. parnassiifolius*.

Baltisberger (1992) suggested that *R. wettsteinii* would be of hybrid origin involving either tetraploid *R. montenegrinus* or diploid *R. seguieri* as parental lineages. Our study strongly supports the hypothesis of an allopolyploid origin for *R. wettsteinii*, and determines with confidence the progenitors. A hybrid species generally inherits the nuclear DNA of both progenitors and plastid DNA of only one (commonly maternal parent). In our analyses, all the plastid accessions (*rpl32-trnL*, *rps16-trnQ*, *trnK-matK*, *ycf6-psbM*) of *R. wettsteinii* cluster with *R. parnassiifolius* as a sister clade (Fig. 2). Therefore our result leads us to propose *R. parnassiifolius* (specifically the subspecies tetraploid *"heterocarpus"*) as the maternal progenitor of *R. wettsteinii*. Although the tetraploids of *R. parnassiifolius* subsp. *heterocarpus* reproduce mostly via pseudogamous apomixis (Cires et al. 2010), a study on pollen malformation and ploidy levels in *R. parnassiifolius s.l.* (Cires et al. 2011) demonstrated pollen viability in these tetraploid populations. Furthermore, and due to its topological position in the ITS phylogenetic trees, we can also suggest the tetraploid *R. montenegrinus* as a potential parent (paternal) of *R. wettsteinii* (Figs. 1, 3). In both cases, the fusion of 2x (reduced pollen gametes) would explain the origin of *R. wettsteinii*. The fusion of an unreduced gamete with a haploid gamete ("triploid bridge"), and a posterior self-fertilisation or backcrossing to diploids has been proposed as the most extended route to allopolyploid speciation (Ramsey and Schemske 1998; Husband 2000). An alternative route would be by fusion of two unreduced gametes, mainly in dense hybrid zones, marginal or disturbed habitats and/or other limiting conditions (Thompson and Lumaret 1992; Ramsey and Schemske 1998). Although this second pathway was thought to be rare (Mallet 2007), it has been found with much greater frequency than expected (Paun et al. 2009; Chase et al. 2010). Nevertheless, the possibility of an unsampled paternal parent cannot be discarded.

An origin of the main *Ranunculus* lineages in the late Miocene and their diversification in the Pliocene was suggested (Paun et al. 2005; Emadzade and Hörandl 2011), which means pre-glacial divergence events. Polyploidy is a trait often correlated with the degree of glaciation (Stebbins 1984) inasmuch as multiple genomic complements are predominant in plants endemic to formerly glaciated areas (e.g. Brochmann et al. 2003). During Pleistocene glaciations, survival of tree species was likely restricted to small areas with favorable conditions—"refugia within refugia"—as has been hypothesized for the Iberian Peninsula, and also can be the case for the Balkan Peninsula, characterised by a drier and more continental climate (Bardy et al. 2010). On the contrary, polyploidy does not usually happen in the southern European alpine species of the genus *Ranunculus*, since it occurs in only few species, such as *R. kuepferi*, *R. montanus, R. parnassiifolius* and *R. wettsteinii*. The effects of Pleistocene glacial cycles on alpine and arctic regions presumably played a key role in contacting and promoting polyploid speciation (Casazza et al. 2012). Although there are some tetraploid populations in southern Europe, we could neither find relationships between polyploidy and monophyletic groups of populations in the clade of *R. parnassiifolius-R. amplexicaulis* (Fig. 3). Considering that many southerly species of *Ranunculus* are diploids and that polyploidy is rare within the genus, polyploidization events appear to have occurred independently across lineages (e.g. Cires et al. 2012).

Detailed studies based on a molecular approach of those species characterised by a narrow distribution in the mountains of the Balkan Peninsula, are of paramount importance for establishing conservation priorities (e.g. *Androsace komovensis*, Schönswetter and Schneeweiss 2009; *Campanula comosiformis*, Frajman and Schneeweiss 2009). This is also the case of *R. wettsteinii* on Mt. Korab, where further genetic variation analyses as well as possible additional populations search on the neighbouring mountains are required. Even more, little is known about the number of individuals from the single known population of *R. wettsteinii*. This species has been ranked within the "List of higher plant groups of national importance" (Ministry of Environment and Physical Planning 2003) and included in the "IUCN 1997—Red List of Threatened Plants" (Walter and Gillett 1998) as "indeterminate". Moreover, some Botanical Gardens such as the Balkan Botanic Garden of Kroussia have recently formulated a clear conservation strategy, which is targeted primarily at the Important Plant Species such as Balkan endemics of narrow distribution range (Priority 2; see Krigas et al. 2010). Considering this, any further collecting activities must be avoided and ex situ cultivation measures should be undertaken urgently. Finally, studies have to be drawn up concerning the state of the population of this very rare and endemic species, which would provide quantitative data

Fig. 3 Phylogenetic networks analysis (NeighborNet splits graph) based on ITS and plastid regions (*rpl32-trnL*, *rps16-trnQ*, *trnK-matK*, *ycf6-psbM*). Numbers along branches are bootstrap values from 1,000 replicates. The least squares fit index for the split network has a value of 98.62, 99.18 and 97.88 % for ITS, plastid and concatenated data, respectively


A) ITS data
0.001
R. amplexicaulis (2x)¹
R. amplexicaulis (2x)²
R. p. subsp. favargeri (4x)¹
R. p. subsp. favargeri (2x)²
R. p. subsp. favargeri (2x)³
R. p. subsp. favargeri (2x)⁴
R. p. subsp. favargeri (2x)⁵
R. p. subsp. parnassifolius (4x)²
R. p. subsp. parnassifolius (2x)¹
R. p. subsp. heterocarpus (4x)¹
R. p. subsp. heterocarpus (4x)²
R. p. subsp. heterocarpus (4x)³
R. c. subsp. cabrerensis (2x)¹
R. c. subsp. cabrerensis (2x)²
R. c. subsp. cabrerensis (2x)³
R. c. subsp. muniellensis (2x)¹
98.9
R. montenegrinus (5x)²
R. montenegrinus (4x)³
R. montenegrinus (4x)¹
R. wettsteinii (4x)¹
R. wettsteinii (4x)³
R. wettsteinii (4x)²
R. wettsteinii (4x)⁴
89.0
R. seguieri (2x)¹
R. seguieri (2x)²
95.9
R. pyrenaeus (2x)¹
R. pyrenaeus (2x)²
R. pyrenaeus (2x)³
R. gramineus (2x)¹
R. aconitifolius (2x)¹
B) Plastid data
0.001
R. seguieri (2x)¹
R. seguieri (2x)²
R. montenegrinus (4x)¹
R. montenegrinus (5x)²
R. montenegrinus (4x)³
R. aconitifolius (2x)¹
100
R. c. subsp. cabrerensis (2x)¹
R. c. subsp. cabrerensis (2x)²
R. c. subsp. cabrerensis (2x)³
R. c. subsp. muniellensis (2x)¹
R. gramineus (2x)¹
100
100
R. amplexicaulis (2x)¹
R. amplexicaulis (2x)²
R. p. subsp. favargeri (4x)¹
R. p. subsp. favargeri (2x)²
R. p. subsp. favargeri (2x)³
R. p. subsp. favargeri (2x)⁴
R. p. subsp. favargeri (2x)⁵
R. p. subsp. parnassifolius (4x)²
R. p. subsp. parnassifolius (2x)¹
R. p. subsp. heterocarpus (4x)¹
R. p. subsp. heterocarpus (4x)²
R. p. subsp. heterocarpus (4x)³
100
100
R. pyrenaeus (2x)¹
R. pyrenaeus (2x)²
R. pyrenaeus (2x)³
R. wettsteinii (4x)¹
R. wettsteinii (4x)²
R. wettsteinii (4x)³
R. wettsteinii (4x)⁴
C) Concatenated data
0.001
R. c. subsp. cabrerensis (2x)¹
R. c. subsp. cabrerensis (2x)²
R. c. subsp. cabrerensis (2x)³
R. c. subsp. muniellensis (2x)¹
R. p. subsp. favargeri (4x)¹
R. p. subsp. favargeri (2x)²
R. p. subsp. favargeri (2x)³
R. p. subsp. favargeri (2x)⁴
R. p. subsp. favargeri (2x)⁵
R. amplexicaulis (2x)¹
R. amplexicaulis (2x)²
R. p. subsp. heterocarpus (4x)¹
R. p. subsp. heterocarpus (4x)²
R. p. subsp. heterocarpus (4x)³
R. gramineus (2x)¹
R. p. subsp. parnassifolius (4x)²
R. p. subsp. parnassifolius (2x)¹
93.7
100
R. wettsteinii (4x)¹
R. wettsteinii (4x)²
R. wettsteinii (4x)³
R. wettsteinii (4x)⁴
R. aconitifolius (2x)¹
100
R. pyrenaeus (2x)¹
R. pyrenaeus (2x)²
R. pyrenaeus (2x)³
R. seguieri (2x)¹
R. seguieri (2x)²
R. montenegrinus (4x)¹
R. montenegrinus (5x)²
R. montenegrinus (4x)³

regarding the number of specimens and the biological vitality of the population.

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Appendix

Plant materials, voucher information and GenBank accessions for DNA sequences used in this paper. Sequences directly retrieved from GenBank are in italics. Systematics of *Ranunculus parnassiifolius* is based on Cires et al. (2012).

Ranunculus taxa (population code); Locality; Ploidy Level; Collector; Collection number and herbarium; ITS GenBank no.; *rpl32-trnL* GenBank no.; *trnQ-rps16* GenBank no.; *trnK-matK* GenBank no.; *ycf6-psbM* GenBank no.

Ficaria verna Huds. (=*R. ficaria* L.); (1); Villaviciosa (Asturias, Spain); 2n=2x; J.A. Fernández Prieto & J. Homet 31967 (FCO); *JX025227*; *JX025320*; *JX025282*; KF528859; KF528890. *Ranunculus aconitifolius* L.; (1); Somiedo (Asturias, Spain); 2n=2x; E. Cires & J.A. Fernández Prieto 31859 (FCO); *JX025228*; *JX025321*; *JX025283*; KF528860; KF528891. *R. amplexicaulis* L.; (1); Portillo de las Yeguas (Cantabria, Spain); 2n=2x; A. Bueno & E. Cires 31962 (FCO); *JX025229*; *JX025322*; *JX025284*; KF528861; KF528892. (2); Somiedo (Asturias, Spain); 2n=2x; E. Cires & J.A. Fernández Prieto 31963 (FCO); *JX025230*; *JX025323*; *JX025285*; KF528862; KF528893. *R. cabrerensis* Rothm.; (1); Abelgas de Luna (León, Spain); 2n=2x; C. Cuesta & E. Cires 31368 (FCO); *JX025237*; *JX025328*; *JX025290*; KF528863; KF528894. (2); Lago de la Baña (León, Spain); 2n=2x; E. Cires, B. Jiménez-Alfaro & L. González 31369 (FCO); *JX025234*; *JX025327*; *JX025289*; KF528864; KF528895. (3); Portillo de las Yeguas (Cantabria, Spain); 2n=2x; A. Bueno & E. Cires 31371 (FCO); *JX025239*; *JX025330*; *JX025292*; KF528865; KF528896. *R. cabrerensis* subsp. *muniellensis* (Bueno, Fern. Casado & Fern. Prieto) Fern. Prieto & Cires; (1); Muniellos Biosphere Reserve (Asturias, Spain); 2n=2x; E. Cires 31370 (FCO); *JX025271*; *JX025350*; *JX025312*; KF528866; KF528897. *R. gramineus* L.; (1); Somiedo (Asturias, Spain); 2n=2x; E. Cires & J.A. Fernández Prieto 31968 (FCO); *JX025232*; *JX025325*; *JX025287*; KF528867; KF528898. *R. montenegrinus* Lindtner; (1); Dinibeg (Sar Planina, Macedonia); 2n=4x; M. Baltisberger no. 12258 (Z/ZT); KF528832; KF528841; KF528850; KF528868; KF528899. (2); Kom Kucki (Komovi, Montenegro), 2n=5x; M. Baltisberger no. 11444 (Z/ZT); KF528833; KF528842; KF528851; KF528869; KF528900. (3); Mali Titov Vrv (Sar Planina, Macedonia); 2n=4x; M. Baltisberger no. 12269 (Z/ZT); KF528834; KF528843; KF528852; KF528870; KF528901. *R. parnassiifolius* subsp. *favargeri* P. Küpfer; (1); Somiedo (Asturias, Spain); 2n=4x; E. Cires & J.A. Fernández Prieto 31103 (FCO); *JX025247*; *JX025335*; *JX025297*; KF528871; KF528902. (2); Jou de los Boches (Asturias, Spain); 2n=2x; A. Fernández & E. Cires 31105 (FCO); *JX025266*; *JX025346*; *JX025308*; KF528872; KF528903. (3); La Canalona (Cantabria, Spain); 2n=2x; A. Fernández & E. Cires 31107 (FCO); *JX025263*; *JX025344*; *JX025306*; KF528873; KF528904. (4); Pandébano (Asturias, Spain); 2n=2x; A. Fernández & E. Cires 31115 (FCO); *JX025268*; *JX025347*; *JX025309*; KF528874; KF528905. (5); Urriellu (Asturias, Spain); 2n=2x; A. Fernández & E. Cires 31117 (FCO); *JX025270*; *JX025349*; *JX025311*; KF528875; KF528906. *R. parnassiifolius* subsp. *heterocarpus* P. Küpfer; (1); Albulapass (Graubünden, Switzerland); 2n=4x; C. Cuesta, E. Cires, M. Ceballos & J.A. Fernández Prieto 31353 (FCO); *JX025243*; *JX025332*; *JX025294*; KF528876; KF528907. (2); Cortina d'Ampezzo (Belluno, Italy); 2n=4x; C. Cuesta, E. Cires, M. Ceballos & J.A. Fernández Prieto 31354 (FCO); *JX025245*; *JX025334*; *JX025296*; KF528877; KF528908. (3); Grand Chavalard (Valais, Switzerland); 2n=4x; C. Cuesta, E. Cires, M. Ceballos & J.A. Fernández Prieto 31366 (FCO); *JX025241*; *JX025331*; *JX025293*; KF528878; KF528909. *R. parnassiifolius* subsp. *parnassiifolius* L.; (1); Col d'Arlas (Aquitaine, France); 2n=2x; E. Cires & J.A. Fernández Prieto 31355 (FCO); *JX025265*; *JX025345*; *JX025307*; KF528879; KF528910. (2); Chisagües (Huesca, Spain); 2n=4x; E. Cires & J.A. Fernández Prieto 31360 (FCO); *JX025253*; *JX025339*; *JX025301*; KF528880; KF528911. *R. pyrenaeus* L.; (1); Chisagües (Huesca, Spain); 2n=2x; E. Cires & J.A. Fernández Prieto 31971 (FCO); *JX025276*; *JX025353*; *JX025315*; KF528881; KF528912. (2); Pas de la Casa (Encamp, Andorra); 2n=2x; E. Cires & J.A. Fernández Prieto 31372 (FCO); *JX025277*; *JX025354*; *JX025316*; KF528882; KF528913. (3); Fontalba (Gerona, Spain); 2n=2x; E. Cires & J.A. Fernández Prieto 31972 (FCO); *JX025278*; *JX025355*; *JX025317*; KF528883; KF528914. *R. seguieri* Vill.; (1); M. Vettore, (NE of Rome, C Italy); 2n=2x; M. Baltisberger no. 12857 (Z/ZT); KF528835; KF528844; KF528853; KF528884; KF528915. (2); Feltre, (E of Trento, N Italy); 2n=2x; M. Baltisberger no. 12056 (Z/ZT); KF528836; KF528845; KF528854; KF528885; KF528916. *R. wettsteinii* Dörfl.; (1, identified as *R. parnassiifolius* from the Balkan Peninsula by M. Baltisberger); Kula Ziberit (Korab, Albania-Macedonia); 2n=4x; M. Baltisberger no. 12421 (Z/ZT); KF528837; KF528846; KF528855; KF528886; KF528917. (2, identified as *R. parnassiifolius* from the Balkan Peninsula by M. Baltisberger); Kula Ziberit (Korab, Albania-Macedonia); 2n=4x; M. Baltisberger s.n.; KF528838; KF528847; KF528856; KF528887; KF528918. (3, identified as *R. wettsteinii* from the Balkan Peninsula by M. Baltisberger); Kula Ziberit (Korab, Albania-Macedonia); 2n=4x; M. Baltisberger no. 12426 (Z/ZT); KF528839; KF528848; KF528857; KF528888; KF528919. (4, identified as *R. wettsteinii* from the Balkan Peninsula by M. Baltisberger); Kula Ziberit (Korab, Albania-Macedonia); 2n=4x; M. Baltisberger s.n.; KF528840; KF528849; KF528858; KF528889; KF528920.

References

Albach, D. C., von Sternburg, M., Scalone, R., & Bardy, K. E. (2009). Phylogenetic analysis and differentiation of *Veronica* subgenus *Stenocarpon* in the Balkan Peninsula. *Botanical Journal of the Linnean Society, 159*, 616–636.

Baltisberger, M. (1992). Botanische Notizen und zytologische Untersuchungen an einigen Pflanzen (insbesondere aus den Gattungen *Ranunculus* und *Achillea*) aus dem albanisch-jugoslawischen Grenzgebiet (Korab, Sar Planina). *Berichte des Geobotanischen Institutes der Eidgenössische Technische Hochschule, Stiftung Rübel, Zürich 58*, 192–211.

Bardy, K. E., Albach, D. C., Schneeweiss, G. M., Fischer, M. A., & Schönswetter, P. (2010). Disentangling phylogeography, polyploid evolution and taxonomy of a woodland herb (*Veronica chamaedrys* group, Plantaginaceae s.l.) in southeastern Europe. *Molecular Phylogenetics and Evolution, 57*, 771–786.

Bennett, K. D., Tzedakis, P. C., & Willis, K. J. (1991). Quaternary refugia of north European trees. *Journal of Biogeography, 18*, 103–115.

Bittkau, C., & Comes, H. P. (2005). Evolutionary processes in a continental island system: molecular phylogeography of the Aegean *Nigella arvensis* alliance (Ranunculaceae) inferred from chloroplast DNA. *Molecular Ecology, 14*, 4065–4083.

Brochmann, C., Gabrielsen, T. M., Nordal, I., Landvik, J. Y., & Elven, R. (2003). Glacial survival or tabula rasa? The history of North Atlantic biota revisited. *Taxon, 52*, 417–450.

Casazza, G., Granato, L., Minuto, L., & Conti, E. (2012). Polyploid evolution and Pleistocene glacial cycles: a case study from the alpine primrose *Primula marginata* (Primulaceae). *BMC Evolutionary Biology, 12*, 56.

Chase, M. W., Paun, O., & Fay, M. F. (2010). Hybridization and speciation in angiosperms: a role for pollinator shifts? *BMC Biology, 8*, 45.

Cires, E., & Fernández Prieto, J. A. (2012). The Iberian endemic species *Ranunculus cabrerensis* Rothm.: an intricate history in the *Ranunculus parnassiifolius* L. polyploid complex. *Plant Systematics and Evolution, 298*, 121–138.

Cires, E., Cuesta, C., Peredo, E. L., Revilla, M. A., & Fernández Prieto, J. A. (2009). Genome size variation and morphological differentiation within *Ranunculus parnassifolius* group (Ranunculaceae) from calcareous screes in the Northwest of Spain. *Plant Systematics and Evolution, 281*, 193–208.

Cires, E., Cuesta, C., Revilla, M. A., & Fernández Prieto, J. A. (2010). Intraspecific genome size variation and morphological differentiation of *Ranunculus parnassifolius* (Ranunculaceae), an Alpine-Pyrenean-Cantabrian polyploid group. *Botanical Journal of the Linnean Society, 101*, 251–271.

Cires, E., Cuesta, C., Fernández Casado, M. A., Fernández-Carvajal, M. C., & Fernández Prieto, J. A. (2011). Malformación polínica y niveles de ploidía en *Ranunculus* grex *parnassiifolius*. *Acta Botanica Malacitana, 36*, 145–158.

Cires, E., Cuesta, C., Vargas, P., & Fernández Prieto, J. A. (2012). Unravelling the evolutionary history of the polyploid complex *Ranunculus parnassiifolius* (Ranunculaceae). *Botanical Journal of the Linnean Society, 107*, 477–493.

Dolphin, K., Belshaw, R., Orme, C. D. L., & Quicke, D. L. J. (2000). Noise and incongruence: interpreting results of the incongruence length difference test. *Molecular Phylogenetics and Evolution, 17*, 401–406.

Dörfler, I. (1918). *Ranunculus wettsteinii* Dörfler spec. nova. *Anzeiger der Kaiserliche Akademie der wissenschaften in Wien–Mathematisch-naturwissenschaftliche Klasse 55*. 282.

Dumolin-Lapègue, S., Pemonge, M.-H., & Petit, R. J. (1997). An enlarged set of consensus primers for the study of organelle DNA in plant. *Molecular Ecology, 6*, 393–397.

Ehlers, J., & Gibbard, P. L. (2003). Extent and chronology of glaciations. *Quaternary Science Reviews, 22*, 1561–1568.

Emadzade, K., & Hörandl, E. (2011). Northern Hemisphere origin, transoceanic dispersal, and diversification of Ranunculeae DC. (Ranunculaceae) in the Cenozoic. *Journal of Biogeography, 38*, 517–530.

Emadzade, K., Lehnebach, C., Lockhart, P., & Hörandl, E. (2010). A molecular phylogeny, morphology and classification of genera of Ranunculeae (Ranunculaceae). *Taxon, 59*, 809–828.

Farris, J. S., Källersjö, M., Kluge, A. G., & Bult, C. (1995). Testing significance of incongruence. *Cladistics, 10*, 315–319.

Frajman, B., & Oxelman, B. (2007). Reticulate phylogenetics and phytogeographical structure of *Heliosperma* (Sileneae, Caryophyllaceae) inferred from chloroplast and nuclear DNA sequences. *Molecular Phylogenetics and Evolution, 43*, 140–155.

Frajman, B., & Schneeweiss, G. M. (2009). A Campanulaceous fate: the Albanian stenoendemic *Asyneuma comosiforme* in fact belongs to isophyllous *Campanula*. *Systematic Botany, 34*, 595–601.

Frodin, D. G. (2004). History and concepts of big plant genera. *Taxon, 53*, 753–776.

Gaston, K. J., & David, R. (1994). Hotspots across Europe. *Biodiversity Letters, 2*, 108–116.

Hayek, A. (1924). Zweiter Beitrag zur Kenntnis der Flora von Albanien. *Anzeiger der Kaiserliche Akademie der wissenschaften in Wien–Mathematisch-naturwissenschaftliche Klasse 99*, 101–223.

Hewitt, G. M. (2000). The genetic legacy of the Quaternary iceages. *Nature, 405*, 907–913.

Hipp, A. L., Hall, J. C., & Sytsma, K. J. (2004). Phylogenetic accuracy, congruence between data partitions, and performance of the ILD. *Systematic Biology, 53*, 81–89.

Hörandl, E., & Emadzade, K. (2012). Evolutionary classification: a case study on the diverse plant genus *Ranunculus* L. (Ranunculaceae). *Perspectives in Plant Ecology Evolution and Systematics, 14*, 310–324.

Hörandl, E., Paun, O., Johansson, J. T., Lehnebach, C., Armstrong, T., Chen, L., et al. (2005). Phylogenetic relationships and evolutionary traits in *Ranunculus* s.l. (Ranunculaceae) inferred from ITS sequence analysis. *Molecular Phylogenetics and Evolution, 36*, 305–327.

Huber, W. (1988). Natürliche Bastardierungen zwischen weissblühenden *Ranunculus*-Arten in den Alpen. *Veröffentlichungen des Geobotanischen Institutes der ETH Zürich, Stiftung Rübel 100*, 1–160.

Husband, B. C. (2000). Constraints on polyploid evolution: a test of the minority cytotype exclusion principle. *Proceedings of the Royal Society B, 267*, 217–223.

Huson, D. H., & Bryant, D. (2006). Application of phylogenetic networks in evolutionary studies. *Molecular Biology and Evolution, 23*, 254–267.

Ibrahim, K. M., Nichols, R. A., & Hewitt, G. M. (1996). Spatial patterns of genetic variation generated by different forms of dispersal during range expansion. *Heredity, 77*, 282–291.

Johnson, L. A., & Soltis, D. E. (1994). *matK* DNA sequences and phylogenetic reconstruction in Saxifragaceae s.s. *Systematic Botany, 19*, 143–156.

Krigas, N., Mouflis, G., Grigoriadou, K., & Maloupa, E. (2010). Conservation of important plants from the Ionian Islands at the Balkan Botanic Garden of Kroussia, N Greece: linking with GIS the *in situ* collection data with plant propagation and the *ex situ* cultivation. *Biodiversity and Conservation, 19*, 3583–3603.

Kryštufek, B., & Reed, J. M. (2004). Pattern and process in Balkan biodiversity—an overview. In H. I. Griffiths, B. Kryštufek, & J. M. Reed (Eds.), *Balkan biodiversity—pattern and process in the European hotspot* (pp. 1–8). Dordrecht: Kluwer.

Kučera, J., Tremetsberger, K., Vojta, J., & Marhold, K. (2008). Molecular study of the *Cardamine maritima* group (Brassicaceae) from the Balkan and Apennine Peninsulas based on amplified fragment length polymorphism. *Plant Systematics and Evolution, 275*, 193–207.

Lo Presti, R. M., Oppolzer, S., & Oberprieler, C. (2010). A molecular phylogeny and a revised classification of the Mediterranean genus *Anthemis s.l.* (Compositae, Anthemideae) based on three molecular markers and micromorphological characters. *Taxon, 59*, 1441–1456.

Magyari, E. K., Chapman, J. C., Gaydarska, B., Marinova, E., Deli, T., Huntley, J. P., et al. (2008). The 'oriental' component of the Balkan flora: evidence of its presence on the Thracian Plain during the Weichselian late-glacial. *Journal of Biogeography, 35*, 865–883.

Mallet, J. (2007). Hybrid speciation. *Nature, 446*, 279–283.

Mereďa, P., Jr., Hodálová, I., Kučera, J., Zozomová-Lihová, J., Letz, D. R., & Slovák, M. (2011). Genetic and morphological variation in *Viola suavis s.l.* (Violaceae) in the western Balkan Peninsula: two endemic subspecies revealed. *Systematics and Biodiversity, 9*, 211–231.

Ministry of Environment and Physical Planning (2003). Country study for biodiversity of the Republic of Macedonia (First National Report), Skopje.

Nei, M., & Kumar, S. (2000). *Molecular evolution and phylogenetics*. New York: Oxford University Press.

Nylander, J. A. A. (2004). MrModeltest v2. Program distributed by the author. Evolutionary Biology Centre, Uppsala University. Available at: http://www.abc.se/~nylander/.

Park, J.-M., Kovačić, S., Liber, Z., Eddie, W. M. M., & Schneeweiss, G. M. (2006). Phylogeny and biogeography of isophyllous species of *Campanula* (Campanulaceae) in the Mediterranean area. *Systematic Botany, 31*, 862–880.

Paun, O., Lehnebach, C., Johansson, J. T., Lockhart, P., & Hörandl, E. (2005). Phylogenetic relationships and biogeography of *Ranunculus* and allied genera (Ranunculaceae) in the Mediterranean region and in the European alpine system. *Taxon, 54*, 911–930.

Paun, O., Forest, F., Fay, M. F., & Chase, M. W. (2009). Hybrid speciation in angiosperms: parental divergence drives ploidy. *New Phytologist, 182*, 507–518.

Rambaut, A., & Drummond, A. J. (2007). Tracer v1.5. See http://beast.bio.ed.ac.uk/Tracer.

Ramsey, J., & Schemske, D. W. (1998). Pathways, mechanisms, and rates of polyploid formation in flowering plants. *Annual Review of Ecology and Systematics, 29*, 467–501.

Ronquist, F., Teslenko, M., van der Mark, P., Ayres, D. L., Darling, A., Höhna, S., et al. (2012). MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. *Systematic Biology, 61*, 539–542.

Schönswetter, P., & Schneeweiss, G. M. (2009). *Androsace komovensis* sp. nov., a long mistaken local endemic from the southern Balkan Peninsula with biogeographic links to the Eastern Alps. *Taxon, 58*, 544–549.

Shaw, J., Lickey, E. B., Beck, J. T., Farmer, S. B., Liu, W., Miller, J., et al. (2005). The tortoise and the hare II: relative utility of 21 noncoding chloroplast DNA sequences for phylogenetic analysis. *American Journal of Botany, 92*, 142–166.

Shaw, J., Lickey, E. B., Schilling, E. E., & Small, R. L. (2007). Comparison of whole chloroplast genome sequences to choose noncoding regions for phylogenetic studies in angiosperms: the tortoise and the hare III. *American Journal of Botany, 94*, 275–288.

Slovák, M., Urfus, T., Vít, P., & Marhold, K. (2009). The Balkan endemic *Picris hispidissima* (Compositae): morphology, nuclear DNA content and relationship to the polymorphic *P. hieracioides*. *Plant Systematics and Evolution, 278*, 187–201.

Stebbins, G. L. (1984). Polyploidy and the distribution of the arctic-alpine flora: new evidence and a new approach. *Botanica Helvetica, 94*, 1–14.

Sun, Y., Skinner, D. Z., Liang, G. H., & Hulbert, S. H. (1994). Phylogenetic analysis of *Sorghum* and related taxa using internal transcribed spacers of nuclear ribosomal DNA. *Theoretical and Applied Genetics, 89*, 26–32.

Surina, B., Schönswetter, P., & Schneeweiss, G. M. (2011). Quaternary range dynamics of ecologically divergent species (*Edraianthus serpyllifolius* and *E. tenuifolius*, Campanulaceae) within the Balkan refugium. *Journal of Biogeography, 38*, 1381–1393.

Swofford, D. L. (2002). *PAUP* Phylogenetic Analysis Using Parsimony (*and Other Methods). Version 4*. Sunderland. Massachusetts: Sinauer Associates.

Taberlet, P., Fumagalli, L., Wust-Saucy, A.-G., & Cosson, J.-F. (1998). Comparative phylogeography and postglacial colonization routes in Europe. *Molecular Ecology, 7*, 453–464.

Tamura, M. (1995). Angiospermae. Ordnung Ranunculales. Fam. Ranunculaceae. II. Systematic Part. In P. Hiepko (Ed.), *Die natürliche Pflanzenfamilien, ed. 2, 17aIV* (pp. 223–519). Berlin: Duncker & Humblot.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., & Kumar, S. (2011). MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance and maximum parsimony methods. *Molecular Biology and Evolution, 28*, 2731–2739.

Thompson, J. D., & Lumaret, R. (1992). The evolutionary dynamics of polyploid plants: origins, establishment and persistence. *Trends in Ecology & Evolution, 7*, 302–307.

Tutin, T. G., & Akeroyd, J. R. (1993). *Ranunculus* L. In T. G. Tutin, N. A. Burges, A. O. Chater, J. R. Edmondson, V. H. Heywood, D. M. Moore, D. H. Valentine, S. M. Walters, & D. A. Webb (Eds.), *Flora Europaea. Vol. 1. Psilotaceae to Platanaceae* (2nd ed., pp. 269–286). Cambridge: Cambridge University Press.

Tzedakis, P. C., Lawson, I. T., Frogley, M. R., Hewitt, G. M., & Preece, R. C. (2002). Buffered tree population changes in a Quaternary refugium: evolutionary implications. *Science, 297*, 2044–2047.

Walter, K. S., & Gillett, H. J. (1998). *1997 IUCN Red List of Threatened Plants. Compiled by the World Conservation Monitoring Centre* (p. 484). Gland, Switzerland and Cambridge, UK: IUCN—The World Conservation Union.