Journal of Biogeography (J. Biogeogr.) (2011)

ORIGINAL ARTICLE

Quaternary range dynamics of ecologically divergent species (*Edraianthus serpyllifolius* and *E. tenuifolius*, Campanulaceae) within the Balkan refugium

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ABSTRACT

Aim We investigated Quaternary range dynamics of two closely related but ecologically divergent species (cold-tolerant *Edraianthus serpyllifolius* and thermophilic *Edraianthus tenuifolius*) with overlapping distribution ranges endemic to the western Balkan Peninsula, an important yet understudied Pleistocene refugium. Our aims were: to test predictions of the 'refugia-within-refugia' model of strong genetic subdivisions due to population isolation in separate refugia; to explore whether two ecologically divergent species reacted differently to Pleistocene climatic fluctuations; and to test predictions of the displacement refugia model of stronger differentiation among populations in the thermophilic *E. tenuifolius* compared with the cold-tolerant *E. serpyllifolius*.

Location The western Balkan Peninsula.

Methods We gathered amplified fragment-length polymorphism (AFLP) data and plastid DNA sequences from two to five individuals from 10 populations of *E. serpyllifolius* and 22 populations of *E. tenuifolius*, spanning their entire respective distribution areas. AFLP data were analysed using a Bayesian clustering approach and a distance-based network approach. Plastid sequences were used to depict relationships among haplotypes in a statistical parsimony network, and to obtain age estimates in a Bayesian framework.

Results In *E. serpyllifolius*, both AFLP and plastid sequence data showed clear geographic structure. Western populations showed high AFLP diversity and a high number of rare fragments. In *E. tenuifolius*, both markers congruently identified a major phylogeographic split along the lower Neretva valley in central Dalmatia. The most distinct and earliest diverging chloroplast DNA (cpDNA) haplotypes were found further south in the south-easternmost populations. North-western populations, identified as a separate cluster by Bayesian clustering, were characterized by low genetic diversity and a low number of rare AFLP markers.

Main conclusions Clear evidence for multiple Pleistocene refugia is found not only in the high-elevation *E. serpyllifolius*, but also in the lowland *E. tenuifolius*, despite the lack of obvious dispersal barriers, in line with the refugia-within-refugia model. Genealogical relationships and genetic diversity patterns support the hypothesis that cold-adapted *E. serpyllifolius* responded to climatic oscillations mostly by elevational range shifts, whereas thermophilic *E. tenuifolius* did so mainly by latitudinal range shifts, with different phases (and probably extents) of range expansion. In contrast to the displacement refugia hypothesis, the two elevationally differentiated species do not differ in their genetic diversity.

Keywords

Balkan Peninsula, Campanulaceae, Dinaric Alps, dispersal barriers, *Edraianthus*, phylogeography, plant migration, Pleistocene, rear edge, refugium.

Journal of Biogeography

INTRODUCTION

European-wide phylogeographic studies have revealed that the Iberian, Apennine and Balkan peninsulas have acted as major glacial refugia of temperate plant and animal species and provided sources for post-glacial northward range expansion (Ibrahim *et al.*, 1996; Taberlet *et al.*, 1998; Hewitt, 1999; Schmitt, 2007). With the accumulation of phylogeographic studies, it became evident that, within these peninsulas, many lineages show strong genetic subdivisions indicative of population isolation in separate refugia, a concept termed 'refugia within refugia' (Gómez & Lunt, 2007). Whereas this model has been well established for the Iberian Peninsula (Gómez & Lunt, 2007; Horn *et al.*, 2009), data from the Apennine and Balkan peninsulas are scarce (Grassi *et al.*, 2009; Previšić *et al.*, 2009).

Here we test this model in the poorly studied western parts of the Balkan Peninsula – the eastern Adriatic coast and the Dinaric Alps that run north-west–south-east. This region exhibits a high physiographic complexity, with mountain ranges reaching far into the alpine zone, which are separated by deep, often canyon-like valleys with thermophilous submediterranean vegetation penetrating into the peninsula from the coast. This complexity, coupled with the comparatively moderate impact of weak and localized glaciation during Pleistocene climatic fluctuations (Šifrer, 1959; Bognar *et al.*, 1991; Milivojević *et al.*, 2008), should have been conducive to the formation of genetically differentiated groups in regional refugia, as previously suggested for the Iberian Peninsula (Gómez & Lunt, 2007). In some animal species of the western Balkan mountains, phylogeographic splits have been shown to coincide with rivers in deep valleys (e.g. the vole *Dinaromys bogdanovi*, Kryštufek *et al.*, 2007; the newt *Mesotriton alpestris*, Sotiropoulos *et al.*, 2007), but this pattern was not observed in the single mountain plant of the region investigated to date, *Heliosperma pusillum* (Waldst. & Kit.) Rchb. (Frajman & Oxelman, 2007). In contrast, in species with main occurrences at lower elevations in this region, phylogeographic splits are associated with subordinate valley systems (e.g. the *Cardamine maritima* group, Kučera *et al.*, 2008, 2010; the viper *Vipera ammodytes*, Ursenbacher *et al.*, 2008) or with archipelagos such as the Dalmatian Islands (e.g. the lizard *Podarcis melisellensis*, Podnar *et al.*, 2004). Although these data suggest that phylogeographic patterns of low- and high-elevation species in the western Balkan Peninsula may differ, differences among the taxa investigated in traits of potential importance for range shifts, such as dispersal capability, render generalization of the observed patterns difficult.

A good system for study of spatio-temporal diversification patterns on the western Balkan Peninsula is provided by the closely related and co-distributed, but ecologically divergent *Edraianthus serpyllifolius* (Vis.) A. DC. and *Edraianthus tenuifolius* A. DC. (Campanulaceae). *Edraianthus* is a small and phylogenetically tight genus (Stefanović *et al.*, 2008). *Edraianthus* species share floral, fruit and seed morphology (Wettstein, 1887; Janchen, 1910; Lakušić, 1974), suggesting similar pollination and dispersal ecology. Flower visitors in *E. graminifolius* include Hymenoptera, syrphids and Lepidoptera (Makrodimos *et al.*, 2008), although pollinator species are most likely to be restricted to solitary bees and bumblebees of the Hymenoptera, as has been observed in *Campanula* species with similar floral morphology (Blionis & Vokou, 2001). The small seeds are probably dispersed by wind, even though they lack specific adaptations for anemochory (B.S., pers. obs.). After recent taxonomic changes and separation of several

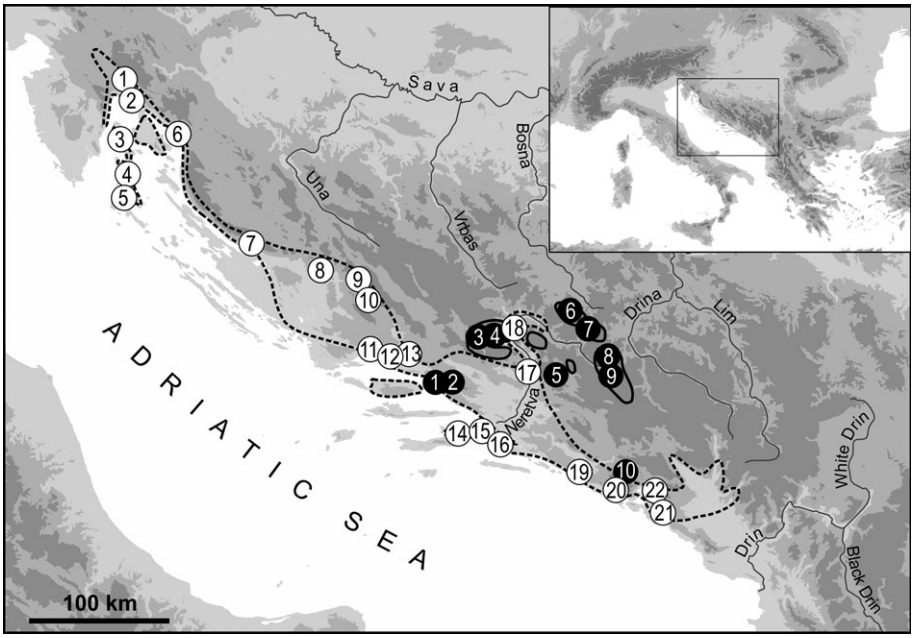

Figure 1 Distribution ranges and sampled populations of *Edraianthus serpyllifolius* (solid line and black circles) and *E. tenuifolius* (hatched line and white circles) on the western Balkan Peninsula. The insert shows the position of the sampling area in south-eastern Europe.

southern populations as distinct taxa (Stefanović *et al.*, 2008; Surina *et al.*, 2009; B.S., unpublished data), *E. serpyllifolius* in its current circumscription is restricted to the central Dinaric Alps (Fig. 1). It is a high-alpine species of north-facing, wet and shaded limestone rock crevices, wind-exposed calcareous grasslands, and snow beds (Lakušić, 1974), only rarely descending to shady river canyons. *Edraianthus tenuifolius* is the second most widespread species of the genus, distributed continuously along the Adriatic coast from south-western Slovenia to northern Albania (Wettstein, 1887; Beck, 1893; Fig. 1). It is a lowland species that inhabits south-facing, dry, rocky, calcareous mediterranean and submediterranean grasslands, and only rarely ascends to the upper montane and subalpine belt (Janchen, 1910). Both species are frequent and locally abundant, but are patchily distributed because the majority of populations are surrounded by unsuitable habitats (dense alpine meadows and forests for *E. serpyllifolius*; forests and scrublands for *E. tenuifolius*; B.S. and P.S., pers. obs.).

The different habitat requirements of *E. serpyllifolius* and *E. tenuifolius* can be utilized to explore whether cold-tolerant and thermophilic species react differently to climatic oscillations (Stewart *et al.*, 2010). For the cold-adapted *E. serpyllifolius*, we expect that deep valleys, which harboured forest or dry steppe vegetation during glacial periods (Šercelj, 1996; Prentice *et al.*, 2000; Ravazzi, 2002; Magri *et al.*, 2006), acted as long-term barriers to dispersal. In contrast to more northerly mountain ranges, such as the much-investigated Alps, the Dinaric Alps were only locally glaciated (Šifrer, 1959; Bognar *et al.*, 1991). Thus elevational range shifts, facilitated by the availability of suitable habitat along the entire elevational gradient over short distances, were a likely response to climatic changes. Genetic data should therefore indicate strong isolation among mountain ranges separated by deep valleys due to limited gene flow (Kryštufek *et al.*, 2007). Although no explicit hypotheses have been put forward for the western Balkan Peninsula, thermophilic species are most likely to have responded to climatic oscillations with latitudinal range shifts (e.g. King & Ferris, 1998; Lunt *et al.*, 1998; Heuertz *et al.*, 2004), probably along the climatically buffered coast, which lacks any obvious dispersal barriers. Southern populations might be stable relict populations, which survived the Quaternary climatic oscillations *in situ*. As such, they should have reduced within-population diversity but should be strongly reciprocally divergent with a disproportionally high degree of genetic differentiation, resulting in high genetic diversity in the rear-edge region (rear-edge hypothesis, Hampe & Petit, 2005). In the leading edge region – the northern part of the distribution area – genetic diversity is expected to be lower due to repeated founder events and rapid range occupation starting from long-distance colonizers (leading-edge hypothesis; Hewitt, 2000). Previous studies have shown that population differentiation is usually weak in recently colonized areas (Schönswetter *et al.*, 2005; Ehrich *et al.*, 2007; but see Ibrahim *et al.*, 1996).

On a range-wide scale, the displacement refugia model, formulated for co-distributed but elevationally differentiated mountain taxa, predicts that high-elevation species faced range fragmentation mostly in the warmer but short interglacial periods, whereas range fragmentation for low-elevation species occurred mostly during the cold and long glacial periods (Kropf *et al.*, 2003). Consequently, low-elevation species are expected to show a higher degree of genetic differentiation and stronger phylogeographic signal than high-elevation species, as was shown in the southern European (mostly) non-alpine *Anthyllis montana* and the alpine *Pritzelago alpina* (Kropf *et al.*, 2002, 2003). A test of this model using closely related species with similar pollination and dispersal syndromes is, however, lacking so far.

Our aim is to investigate Quaternary range dynamics of two closely related, but ecologically divergent species with overlapping distribution ranges on the western Balkan Peninsula, an important yet understudied refugium. To this end, we analysed chloroplast DNA (cpDNA) sequences, which are maternally inherited in most angiosperms (Korpelainen, 2004), including the Campanulaceae (Harris & Ingram, 1991), as well as amplified fragment length polymorphism markers (AFLPs), which are biparentally inherited and derived essentially from the nuclear genome (Bussell *et al.*, 2005), sampling populations of *E. tenuifolius* and *E. serpyllifolius* across their entire distribution areas. First, we tested predictions of the refugia-within-refugia model of strong genetic subdivisions due to population isolation in separate refugia. Specifically, for the cold-tolerant *E. serpyllifolius* we expect that deep phylogeographic splits occur along major river valleys, as has been observed for mountain animals (Kryštufek *et al.*, 2007; Sotiropoulos *et al.*, 2007), whereas no such associations are expected for the thermophilic *E. tenuifolius*. Second, we tested the hypothesis that the two ecologically divergent species reacted differently to Pleistocene climatic fluctuations (Stewart *et al.*, 2010); specifically that the cold-tolerant *E. serpyllifolius* responded via elevational range shifts, whereas the thermophilic *E. tenuifolius* responded via latitudinal range shifts. In the latter, we expect contrasting patterns of genetic diversity in the rear versus the leading edge of the distribution range (Hampe & Petit, 2005). Finally, we tested predictions of the displacement refugia model – stronger differentiation among populations in the thermophilic *E. tenuifolius* compared with the cold-tolerant *E. serpyllifolius*.

MATERIALS AND METHODS

Sampling

Leaf material from two to five individuals per population was sampled along the distribution ranges of the two taxa and dried in silica gel. Due to differently sized distribution ranges (Fig. 1), but comparable sampling densities, the numbers of populations sampled for the two species differed (*E. serpyllifolius*, 10 populations; *E. tenuifolius*, 22 populations; Figs 2a & 3a, respectively). Voucher specimens are deposited at the herbaria of the University of Vienna, Austria (WU), the Natural History Museum Rijeka, Croatia (NHMR), and the University of

Zagreb, Croatia (ZA). Details of sampling localities are given in Table 1 and their locations are shown in Fig. 1.

Laboratory methods

Total genomic DNA was extracted from similar amounts of dried tissue (*c.* 10 mg) following the cetyl trimethyl ammonium bromide (CTAB) protocol (Doyle & Doyle, 1987) with some modifications (Schönswetter *et al.*, 2002). The quality of the extracted DNA was checked on 1% TAE-agarose gels. The AFLP procedure followed Schönswetter *et al.* (2009). One negative control sample was included to test for systematic contamination, and eight samples of *E. serpyllifolius* and 13 of *E. tenuifolius* were replicated to test for reproducibility (Bonin *et al.*, 2004). An initial screening of selective primers was performed using 12 primer combinations. The three final primer combinations for the selective polymerase chain reaction (PCR) were (fluorescent dye in brackets): *Eco*RI (6-Fam)–ACA/*Mse*I–CAT; *Eco*RI (VIC)–ACG/*Mse*I–CAA; *Eco*RI (NED)–ACC/*Mse*I–CAG. Samples (5 μL) of each selective PCR product were purified as described in Schönswetter *et al.* (2009); 1.2 μL of the elution product was combined with 10 μL formamide and 0.1 μL GeneScan ROX (Applied Biosystems, Foster City, CA, USA) and separated on an ABI 3130xl Genetic Analyzer automated capillary sequencer (Applied Biosystems).

Three regions of the chloroplast genome were sequenced: the *trn*G_{UCC}–*trn*R_{UCU} intergenic spacer using primers ccmp3f (Weising & Gardner, 1999) and trnRr (Dumolin-Lapegue *et al.*, 1997); the *rpl*32–*trn*L_{UAG} intergenic spacer using primers rpl23-F and trnL(UAG) (both Shaw *et al.*, 2007); and the *trn*T_{UGU}–*trn*L_{UAA}–*trn*F_{GAA} intergenic spacers including the *trn*L_{UAA} intron using primers a and f (Taberlet *et al.*, 1991). PCR conditions for the first primer pair were 5 min at 95 °C followed by 35 cycles of 1 min at 94 °C, 1 min at 60 °C and 1 min at 72 °C, followed by 10 min at 72 °C. For the second primer pair, the conditions were 5 min at 94 °C followed by 36 cycles of 30 s at 94 °C, 30 s at 50 °C and 2 min at 65 °C, followed by 8 min at 65 °C. Reaction volumes for both primer pairs were 25 μL, comprising 9 μL REDTaq ReadyMix PCR Reaction Mix (Sigma-Aldrich, Vienna, Austria), 1 μL template DNA of unknown concentration and primers at final concentration of 0.2 μM. For the *trn*T–*trn*F region, we followed the recommendations of Borsch *et al.* (2003) with some modifications: 5 min at 95 °C, followed by 35 cycles of 30 s at 94 °C, 30 s at 55 °C and 4 min at 65 °C, followed by 10 min at 65 °C. PCR products were purified using *Escherichia coli* exonuclease I and calf intestine alkaline phosphatase (CIAP; MBI-Fermentas, St Leon-Rot, Germany) according to the manufacturer's instructions. Cycle sequencing was performed using BigDye Terminator chemistry (Applied Biosystems) according to the manufacturer's instructions, the *trn*T–*trn*F region being sequenced in three parts using primers a, c and f (Taberlet *et al.*, 1991), after which electrophoresis was carried out with an ABI 3130xl Genetic Analyzer capillary sequencer (Applied Biosystems). Sequences were assembled using SeqMan II 5.05 (DNAStar Inc., Madison, WI, USA), and aligned by eye using BioEdit 7.0.4.1 (Hall, 1999).

Data analysis

Raw AFLP data were collected and aligned with the internal size standard using ABI Prism GeneScan 3.7.1 (Applied Biosystems). Subsequently, the GeneScan files were imported into Genographer 1.6.0 (Montana State University; version no longer available) for scoring the fragments. The results of scoring were exported as a presence/absence matrix. Using SplitsTree 4.8 (Huson & Bryant, 2006), a NeighborNet diagram, which is well suited to depict reticulate relationships, was produced from Nei–Li distances (Nei & Li, 1979) calculated with TreeCon 1.3b (Van de Peer & De Wachter, 1997). A neighbour-joining analysis was conducted and bootstrapped (1000 pseudoreplicates) with the same program. Nei's (1987) gene diversity was calculated for each population with the R script AFLPdat (Ehrich, 2006; http://www2.uit.no/ikbViewer/page/ansatte/organisasjon/ansatte/person?p_document_id=41186&p_dimension_id=88165&p_lang=2). The same script was used to calculate frequency down-weighted marker values (DW; Schönswetter & Tribsch, 2005) with the modifications given in Winkler *et al.* (2010).

structure 2.2 with a Bayesian clustering approach developed for dominant markers (Pritchard *et al.*, 2000; Falush *et al.*, 2007) was used with an admixture model with uncorrelated allele frequencies and recessive alleles; using a more restrictive model of correlated allele frequencies gave identical results (data not shown). Ten replicate runs for each *K* (number of groups) ranging from 1 to 10 were carried out at the Bioportal of the University of Oslo (http://www.bioportal.uio.no), using a burn-in of 10^5 iterations followed by 10^6 additional Markov chain Monte Carlo (MCMC) iterations. Similarity among results of different runs for the same *K* was calculated according to Nordborg *et al.* (2005) using structure-sum-2009 (http://www2.uit.no/ikbViewer/page/ansatte/organisasjon/ansatte/person?p_document_id=41186&p_dimension_id=88165&p_lang=2). We identified the optimal number of main groups as the value of *K* when the increase in likelihood started to flatten out; the results of replicate runs were similar, and no empty groups (clusters that have no individuals assigned to them) occurred.

To compare genetic divergence within the two species, we calculated the net number of nucleotide differences (Nei & Li, 1979) among populations of *E. serpyllifolius* ($n = 10$) and of *E. tenuifolius* ($n = 22$) using Arlequin 3.11. The values were rescaled by dividing them by the numbers of scored AFLP markers in order to account for different numbers of fragments per species. Significance of differences between the two species was assessed via a two-sample randomization test with 10,000 permutations using the software rt 2.1 (Manly, 1991).

Statistical parsimony networks were constructed from the concatenated chloroplast sequence data using tcs 1.21 (Clement *et al.*, 2000), treating sequence gaps as fifth character

Table 1 Sampling localities, amplified fragment length polymorphism (AFLP) diversity descriptors and plastid DNA haplotypes for the investigated populations of *Edraianthus serpyllifolius* and *E. tenuifolius* in the western Balkan Peninsula.

Sampling locality	Elevation (m)	Long./Lat.	N_{AFLP}	Nei's GD	DW	H_{cp}	GenBank accession numbers
Edraianthus serpyllifolius							
CRO: Biokovo, Sveti Jure	1700	17.06°/43.33°	3	0.218	8.84	VII (5)	HQ679676–HQ679680; HQ679768–HQ679772; HQ679860–HQ679864
CRO: Biokovo, Sveti Jure	1660	17.08°/43.33°	5	0.173	11.34	VII (5)	HQ679681–HQ679685; HQ679773–HQ679777; HQ679865–HQ679869
BiH: Čvrsnica, Jelenak	1800	17.50°/43.59°	5	0.145	3.98	V (2)	HQ679686–HQ679687; HQ679778–HQ679779; HQ679870–HQ679871
BiH: Čvrsnica, Pločno	2100	17.52°/43.59°	5	0.130	4.51	V (4)	HQ679688–HQ679691; HQ679780–HQ679783; HQ679872–HQ679875
BiH: Velež, Velika Velež	1756	18.07°/43.30°	5	0.139	4.55	VI (4)	HQ679692–HQ679695; HQ679784–HQ679787; HQ679876–HQ679879
BiH: Bjelašnica, Bjelašnica	2043	18.26°/43.70°	5	0.148	4.60	I (3)	HQ679696–HQ679698; HQ679788–HQ679790; HQ679880–HQ679882
BiH: Treskavica, Mali Treskač	1500	18.40°/43.57°	5	0.132	4.60	IV (5)	HQ679699–HQ679703; HQ679791–HQ679795; HQ679883–HQ679887
BiH: Zelengora, Orlovačko jezero	1820	18.54°/43.36°	4	0.153	6.51	I (5)	HQ679704–HQ679708; HQ679796–HQ679800; HQ679888–HQ679892
BiH: Maglić, Maglić	2020	18.54°/43.30°	5	0.151	5.17	III (5)	HQ679709–HQ679713; HQ679801–HQ679805; HQ679893–HQ679897
MNE: Orjen, Zubački kabao	1894	18.54°/42.57°	5	0.136	5.74	I (1), II (1)	HQ679714–HQ679715; HQ679806–HQ679807; HQ679898–HQ679899
Edraianthus tenuifolius							
CRO: Grobnik	535	14.55°/45.38°	5	0.042	1.90	I (2)	HQ679716–HQ679717; HQ679624–HQ679625; HQ679808–HQ679809
CRO: Rijeka	310	14.52°/45.33°	5	0.078	2.35	I (2)	HQ679718–HQ679719; HQ679626–HQ679627; HQ679810–HQ679811
CRO: Cres, Sis	405	14.36°/45.06°	4	0.060	2.58	I (2)	HQ679720–HQ679721; HQ679628–HQ679629; HQ679812–HQ679813
CRO: Cres, Martinščica	180	14.42°/44.80°	4	0.073	2.56	I (2)	HQ679722–HQ679723; HQ679630–HQ679631; HQ679814–HQ679815
CRO: Lošinj, Osorščica	450	14.36°/44.68°	5	0.063	2.52	I (2)	HQ679724–HQ679725; HQ679632–HQ679633; HQ679816–HQ679817
CRO: Tomišina draga	100	14.89°/45.07°	4	0.079	1.83	II (4)	HQ679726–HQ679729; HQ679634–HQ679637; HQ679818–HQ679821
CRO: Velika Paklenica	380	15.48°/44.33°	5	0.144	3.89	I (2)	HQ679730–HQ679731; HQ679638–HQ679639; HQ679822–HQ679823
CRO: Pređena	350	16.08°/44.12°	5	0.181	3.90	I (3)	HQ679732–HQ679734; HQ679640–HQ679642; HQ679824–HQ679826
CRO: Dinara, Uništa	1360	16.41°/44.04°	5	0.106	5.38	I (2)	HQ679735–HQ679736; HQ679643–HQ679644; HQ679827–HQ679828
CRO: Sinjsko polje	405	16.48°/43.90°	3	0.15	4.07	I (3)	HQ679737–HQ679739; HQ679645–HQ679647; HQ679829–HQ679831
CRO: Klis	360	16.53°/43.56°	5	0.088	2.97	I (2)	HQ679740–HQ679741; HQ679648–HQ679649; HQ679832–HQ679833
CRO: Mosor, Veliki Kabao	800	16.62°/43.51°	3	0.175	4.71	I (3)	HQ679742–HQ679744; HQ679650–HQ679652; HQ679834–HQ679836
CRO: Mosor, Gornje Sitno	850	16.62°/43.51°	5	0.136	4.03	I (2)	HQ679745–HQ679746; HQ679653–HQ679654; HQ679837–HQ679838
CRO: Pelješac, Sveti Ilija	800	17.16°/42.99°	3	0.174	4.12	VI (2), VII (1)	HQ679747–HQ679749; HQ679655–HQ679657; HQ679839–HQ679841
CRO: Pelješac, Pijavičino	440	17.37°/42.99°	5	0.139	4.18	V (3)	HQ679750–HQ679752; HQ679658–HQ679660; HQ679842–HQ679844
CRO: Pelješac, Žuljana	180	17.48°/42.89°	5	0.192	5.73	III (1), IV (2)	HQ679753–HQ679755; HQ679661–HQ679663; HQ679845–HQ679847
BiH: Jablanica	525	17.75°/43.63°	5	0.086	3.36	VII (2)	HQ679756–HQ679757; HQ679664–HQ679665; HQ679848–HQ679849
BiH: Stolac	240	17.83°/43.34°	5	0.097	3.66	VII (1), VII (1)	HQ679758–HQ679759; HQ679666–HQ679667; HQ679850–HQ679851
BiH: Visočnik	390	18.18°/42.66°	5	0.109	4.31	IX (2)	HQ679760–HQ679761; HQ679668–HQ679669; HQ679852–HQ679853
MNE: Orjen, Kruševice	910	18.50°/42.54°	5	0.116	3.86	X (2)	HQ679762–HQ679763; HQ679670–HQ679671; HQ679854–HQ679855
MNE: Lovćen, Njeguši	885	18.83°/42.44°	5	0.145	3.52	XI (2)	HQ679764–HQ679765; HQ679672–HQ679673; HQ679856–HQ679857
MNE: Lovćen, Ivanova korita	1215	18.85°/42.38°	5	0.152	4.21	XI (2)	HQ679766–HQ679767; HQ679674–HQ679675; HQ679858–HQ679859

BiH, Bosnia and Herzegovina; CRO, Croatia; MNE, Montenegro. N_{AFLP}, number of individuals investigated with AFLP; Nei's GD, Nei's (1987) gene diversity; DW, frequency down-weighted marker values; H_{CP}, chloroplast DNA (cpDNA) haplotypes derived from concatenated sequences, the number of individuals per haplotype per population is given in parentheses; GenBank accession numbers of *ccmp3*–*trn*R; *trn*T–*trn*F; *rpl*32–*trn*L sequences.

state after recoding indels longer than 1 bp as single base-pair indels and excluding polymorphic mononucleotide repeats longer than 5 bp, as these were highly homoplasious (data not shown).

Phylogenetic analyses were conducted using the approach implemented in BEAST 1.4.8 (Drummond & Rambaut, 2007), as this allows for the genealogical uncertainty due to the stochastic nature of the coalescence process to be taken into account, and does not require potentially misleading outgroups for rooting. Initial analysis using the Bayesian skyline plot (Drummond *et al.*, 2005), the most general demographic model, with different group intervals did not indicate any detectable fluctuations in population size (data not shown). Additionally, a demographic model of constant population size through time received slightly higher marginal likelihoods (determined using TRACER 1.4, http://tree.bio.ed.ac.uk/software/tracer) than the more complex model of the Bayesian skyline plot (data not shown), and consequently the final analyses were conducted using the simpler demographic model. As the best-fit substitution models had low Akaike weights (< 0.25) in MODELTEST 3.6 (Posada & Crandall, 1998), and the set of models with a cumulative Akaike weight > 0.95 mostly included models with two to three substitution rate parameters, we finally used an HKY+Γ model to avoid over-parameterization. Based on published substitution rates for plastid DNA (Smith *et al.*, 2008), the prior distribution of the mutation rate was given as a normal distribution with a mean of 4×10^{-3} per site per million years and a wide standard deviation of half the mean. The age estimates thus obtained will be biased towards older ages due to the time dependency of molecular rates (Ho *et al.*, 2007), even if this effect is of a smaller magnitude than initially anticipated (Debruyne & Poinar, 2009). Nevertheless, due to the low level of sequence variation, the age estimates obtained need to be viewed with appropriate caution. In order to keep the number of parameters to be estimated to a minimum, we used a strict clock rather than a relaxed molecular clock. Based on initial analyses using the Bayesian skyline plot (data not shown), the root node was constrained to a maximum age of 5 Ma. Stationarity of the Markov chain, which was run for 3×10^7 generations, was determined using TRACER 1.4. The first 10% of sampled generations was discarded as burn-in, after which all effective sample size values were > 2000. A second run was conducted to confirm convergence of the Markov chain on the stationary distribution. All parameter estimates were based on these two runs combined (54,000 sampling points).

RESULTS

Edraianthus serpyllifolius

A total of 340 reproducible bands were scored; 12 bands were monomorphic (found in all individuals) and 46 were found in only one individual, or were lacking in a single individual. Those bands were excluded from further analyses. The error rate (according to Bonin *et al.*, 2004) before the exclusion of unreliable characters was 0.7%. The NeighborNet diagram complemented with bootstrap values derived from a neighbour-joining analysis (Fig. 2b) revealed strong differentiation of populations 1 and 2 from the remaining ones. Pairs of neighbouring populations always clustered together with bootstrap support (BS) > 80, but no hierarchical structure was apparent, except for a weak separation of populations 6–9 situated in internal parts of the Dinaric Alps (BS 53). Nei's gene diversity ranged from 0.13 in population 4 to 0.22 in population 1 (Table 1; Fig. 2c), DW from 3.98 in population 3 to 11.34 in population 2 (Table 1; Fig. 2d). The mean rescaled net number of nucleotide differences among populations was 0.110 ± 0.050. According to our criteria, $K = 3$ was selected as the appropriate number of groups in the STRUCTURE analysis (see Appendix S1a in Supporting Information). Population membership coefficients for the three clusters identified are shown in Fig. 2e. Most populations were assigned to a single cluster, but populations 1, 2 and 5 showed admixture between different clusters.

The lengths of the *trn*G–*trn*R, *rpl*32–*trn*L and *trn*T–*trn*F sequences were 402, 751 and 1760 bp, respectively. The alignment was 2913 bp long and comprised 19 variable characters, six of which were nucleotide substitutions and 13 were insertion/deletion events (0.65% variability). Combining the three chloroplast sequences for each individual by concatenation and excluding polymorphic mononucleotide repeats gave a total of seven haplotypes in 40 individuals analysed. Intrapopulational haplotype variation was detected in population 10 (haplotypes I and II; Fig. 2f,g; Table 1). BEAST analysis (Fig. 2h) placed the root between western and eastern populations. The median age (and its 95% highest posterior density interval) of this initial differentiation was estimated to be 0.210 (0.017–2.825) Ma.

Edraianthus tenuifolius

We scored 424 reproducible bands; seven bands were monomorphic and 48 were found in only one individual, or were lacking in a single individual. The two latter categories were excluded from further analyses. The error rate was 0.6%. In the NeighborNet diagram (Fig. 3b), two groups of populations were identified, which also received high support (BS 97%) in the neighbour-joining analysis; one included populations 1–13 from the north-west of the distribution area plus one individual of population 14; the second comprised the south-eastern populations 14–22. In order to be able to exclude the possibility of DNA contamination or mix-up of samples, 29 accessions from populations 1, 6–9 and 11–15 were re-extracted and reprocessed, and their positions in the network were confirmed. Nei's gene diversity ranged from 0.04 in population 1 to 0.19 in population 16 (Table 1; Fig. 3c), DW from 1.79 in population 1 to 5.73 in population 16 (Table 1; Fig. 3d). The mean rescaled net number of nucleotide differences among populations was 0.107 ± 0.042. This is not significantly different from that observed in *E. serpyllifolius* ($P = 0.36$). Our criteria suggested $K = 3$ as the appropriate number of groups in the STRUCTURE analysis (see Appendix S1b). Cluster membership coefficients

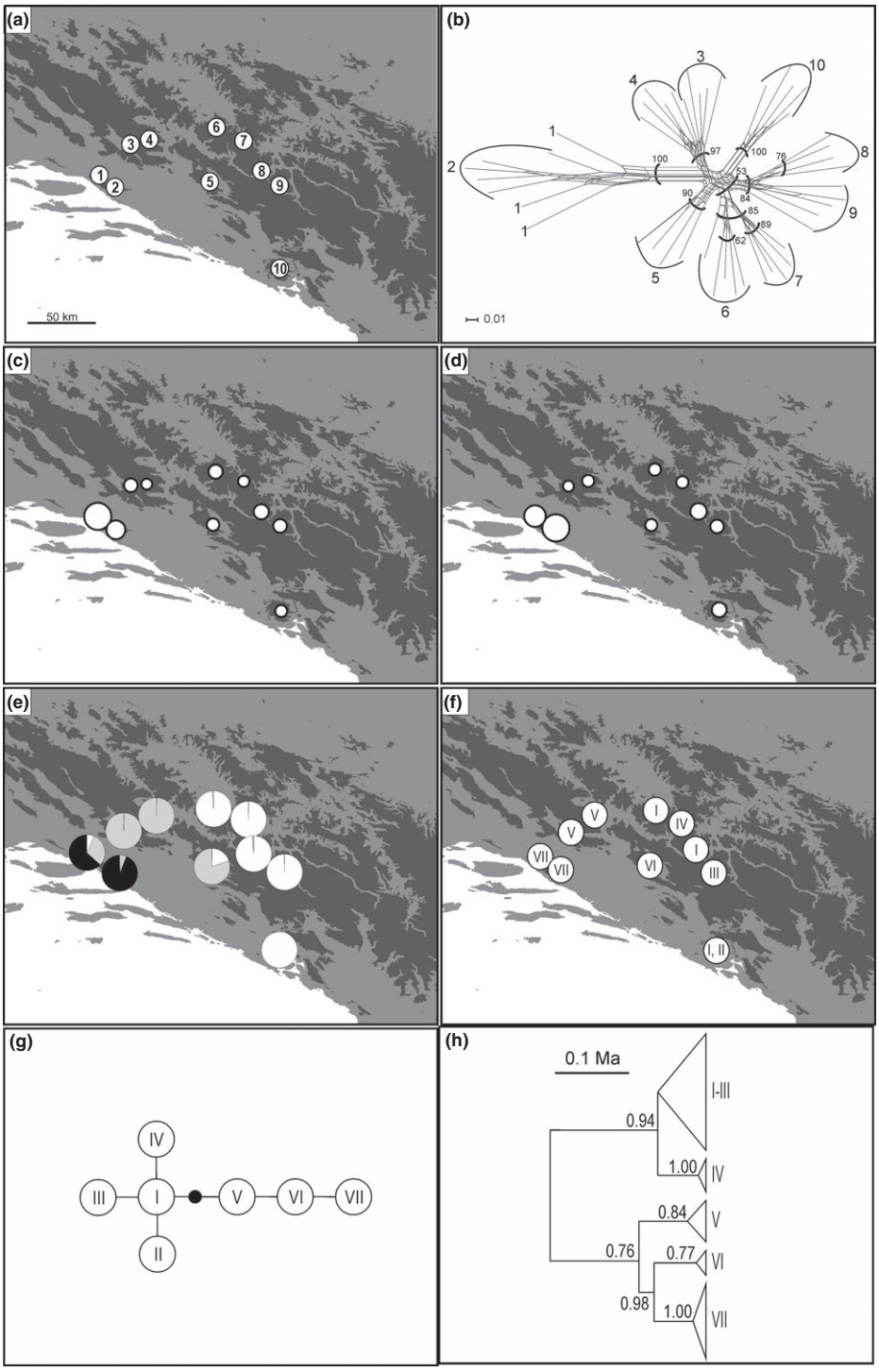

Figure 2 Sampled populations and patterns of amplified fragment length polymorphism (AFLP) and plastid DNA variation of *Edraianthus serpyllifolius* on the western Balkan Peninsula. (a) Sampled populations (see Table 1 for further details). (b) NeighborNet diagram based on a Nei–Li distance matrix (Nei & Li, 1979). Bootstrap values above 50% derived from a neighbour-joining analysis are given for the main branches. (c) Nei's (1987) gene diversity. (d) Frequency down-weighted marker values (Schönswetter & Tribsch, 2005; Winkler *et al.*, 2010). In (c) and (d), the size of the dots is directly proportional to the depicted values. (e) Bayesian clustering of AFLP data using the software STRUCTURE. The three gene pools obtained in the optimal solution are colour-coded (white, grey and black). Populations may be composed of one or several gene-pools. (f) Geographic distribution of plastid DNA haplotypes derived from concatenated sequences of *trn*G_{UCC}–*trn*R_{UCU}, *rpl*32–*trn*L_{UAG} and *trn*T_{UGU}–*trn*L_{UAA}–*trn*F_{GAA} intergenic spacers. (g) Statistical parsimony network of plastid DNA haplotypes. (h) Majority rule consensus tree from strict clock Bayesian analysis with the software BEAST. Node heights correspond to median ages. Numbers along branches are Bayesian posterior probabilities; identical haplotypes or unresolved polytomies are collapsed as triangles, their vertical extension being proportional to the number of individuals.

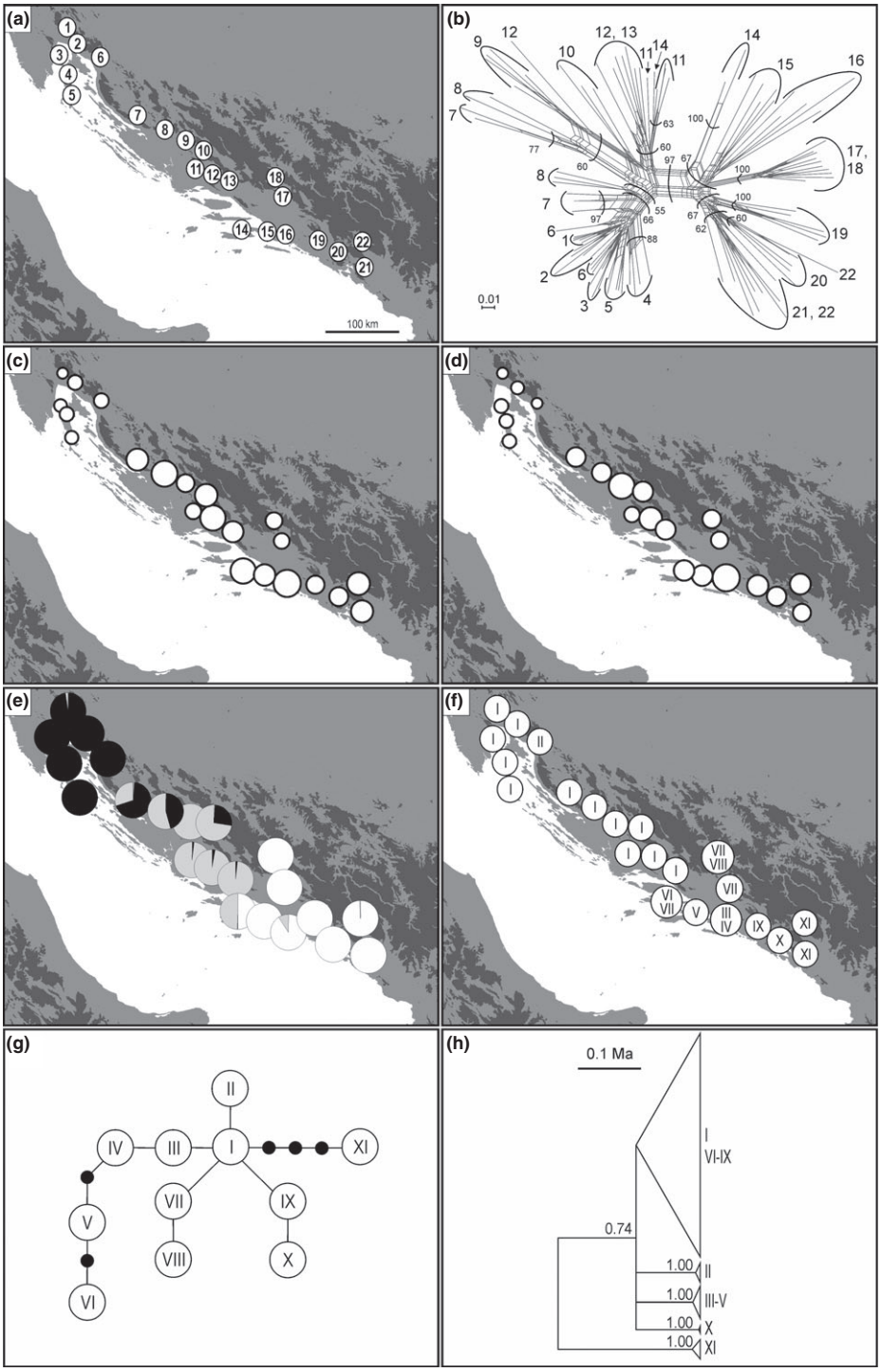

Figure 3 Sampled populations and patterns of amplified fragment length polymorphism (AFLP) and plastid DNA variation of *Edraianthus tenuifolius* on the western Balkan Peninsula. (a) Sampled populations (see Table 1 for further details). (b) NeighborNet diagram based on a Nei–Li distance matrix (Nei & Li, 1979). Bootstrap values above 50% derived from a neighbour-joining analysis of the same matrix are given for the main branches. (c) Nei's (1987) gene diversity. (d) Frequency down-weighted marker values (Schönswetter & Tribsch, 2005; Winkler *et al.*, 2010). In (c) and (d), the size of the dots is directly proportional to the depicted values. (e) Bayesian clustering of AFLP data using the software STRUCTURE. The three gene pools obtained in the optimal solution are colour-coded (white, grey and black). Populations may be composed of one or several gene-pools. (f) Geographic distribution of plastid DNA haplotypes derived from concatenated sequences of *trn*G_{UCC}–*trn*R_{UCU}, *rpl*32–*trn*L_{UAG} and *trn*T_{UGU}–*trn*L_{UAA}–*trn*F_{GAA} intergenic spacers. (g) Statistical parsimony network of plastid DNA haplotypes. (h) Majority rule consensus tree from strict clock Bayesian analysis with the software BEAST. Node heights correspond to median ages. Numbers along branches are Bayesian posterior probabilities; identical haplotypes or unresolved polytomies are collapsed as triangles, their vertical extension being proportional to the number of individuals.

for each population are presented on a geographic basis in Fig. 3e. Most populations are assigned to a single cluster, but populations 7, 8, 10 and 14 showed strong admixture between different clusters.

The lengths of the *trn*G–*trn*R, *rpl*32–*trn*L and *trn*T–*trn*F sequences were 391, 766 and 1792 bp, respectively. The alignment was 2859 bp long and comprised 30 variable characters, nine of which were nucleotide substitutions and 21 were indel events (1.05% variability). After exclusion of polymorphic mononucleotide repeats, the combined sequences gave a total of 11 haplotypes in 52 individuals (Table 1; Fig. 3f,g). Intrapopulational haplotype variation was detected in two populations from the Pelješac Peninsula (population 14, haplotypes VI and VII; population 16, haplotypes III and IV) and in population 18 (haplotypes VII and VIII; Fig. 3f). The most frequent haplotype I was encountered in the north-western populations 1 to 13 (except in population 6). BEAST (Fig. 3h) placed the root between the two easternmost populations [populations 21 and 22, posterior probability (PP) 1] and the remaining ones (PP 0.74), a diversification estimated to be 0.227 (0.017–2.809) Myr old.

DISCUSSION

Refugia-within-refugia on the western Balkan Peninsula

It is becoming increasingly clear that the refugia-within-refugia model developed for the Iberian Peninsula (Gómez & Lunt, 2007) also applies to species of the Balkan region (Podnar *et al.*, 2004; Kryštufek *et al.*, 2007; Ursenbacher *et al.*, 2008). *Edraianthus serpyllifolius* shows genetic differentiation into two (cpDNA; Fig. 2g) to three (AFLP; Fig. 2b,e) geographically distinct groups, which coincide with isolated mountain ranges as also seen in some western Balkan mountain animals (Kryštufek *et al.*, 2007; Sotiropoulos *et al.*, 2007). In contrast to the patterns in animals, however, the lower Neretva valley (separating populations 1–4 from the remaining ones) does not coincide with any major phylogeographic split (Fig. 2e–h), indicating that gene flow has occurred across this deep but narrow valley. Gene exchange between mountain ranges is also evident in population 1 from the Biokovo mountain range, which shows admixture between the local gene pool and that of the closest inland populations 3–4 (Fig. 2e). Both investigated populations from Biokovo (populations 1 and 2) are characterized by elevated levels of gene diversity and rare fragments (Fig. 2b,d). This may be explained by long-term survival of large populations in this climatically favoured area in immediate proximity to the sea. Alternatively, as the Biokovo harbours several *Edraianthus* species, and hybrids involving *E. serpyllifolius* are known (Wettstein in Murbeck, 1891; Gusmus, 1904), the genetic diversity patterns and strong divergence from populations elsewhere may be caused by introgression into *E. serpyllifolius*.

A deep phylogeographic split within *E. tenuifolius*, indicated by AFLP data, separates south-eastern from north-western populations. It coincides with the lowermost Neretva valley (from populations 17 and 18 towards population 15; Fig. 3). As also suggested for the lizard *Podarcis melisellensis* (Podnar *et al.*, 2004), this region is likely to be a secondary contact zone of lineages diversified in phases of geographic isolation. Gene flow between both lineages of *E. tenuifolius* does occur, as is evident from their co-occurrence within the same population (population 14, here probably facilitated by land connections due to lower sea levels in cold periods; Fig. 3b,e), but appears to be rare. This might be due to the lack of suitable habitat in the wide, swampy lowermost Neretva valley hampering migration (Podnar *et al.*, 2004). Alternatively, the secondary contact zone might be of recent origin, and too little time has elapsed for extensive gene flow. The lack of a phylogeographic split along the Neretva valley in other lowland taxa (Ursenbacher *et al.*, 2008) suggests that the mechanism behind the *E. tenuifolius* pattern is not universal.

It is unclear whether the north-western genetic group identified from the AFLP data in *E. tenuifolius* (Fig. 3e) indicates a refugium around the Kvarner Bay. If so, the low levels of AFLP and haplotype diversity would be caused by strong bottlenecks expected for a thermophilic species in a northern refugium, and the admixture of northern and central gene pools in populations 7 and 8 (Fig. 3e) would be the result of secondary contact after post-glacial range expansion (Vallianatos *et al.*, 2001; Zamudio & Savage, 2003; Heilveil & Berlocher, 2006; Liepelt *et al.*, 2009). The lack of distinct northern haplotypes (Fig. 3f), however, rather suggests that the identification of a third group in the AFLP data by STRUCTURE is an artefact of the low genetic diversity in this region caused by founder events during post-glacial range expansion. The latter hypothesis is supported by studies carried out by Podnar *et al.* (2004) on a species of lizard and by Ursenbacher *et al.* (2008) on a species of viper: neither study found any evidence for a refugium that far north.

Different responses to Pleistocene climatic fluctuations

The hypothesis of climate-induced elevational oscillation in mountain plants as compared with latitudinal migration in lowland taxa is supported by several lines of evidence. In the cold-adapted *E. serpyllifolius*, both AFLP and plastid sequence data show clear geographic structure, with gene flow mainly among neighbouring populations within the same mountain range (Fig. 2b,h), as expected if range shifts occurred mostly elevationally (Kryštufek *et al.*, 2007). The main phylogeographic split might date as far back as the late Pliocene (estimated age of the initial diversification is 0.02–2.83 Ma), indicating that the geographic structure has been retained over several cycles of Pleistocene range shifts. In the thermophilic *E. tenuifolius*, north-western populations show reduced AFLP diversity (Fig. 3c) and reduced levels of rare fragments (Fig. 3d). Plastid haplotype diversity in north-western and central populations (populations 1–13) is low, with only two haplotypes separated by a single mutational step (Fig. 3f,g).

Both patterns observed fitted expectations for areas colonized from more southerly regions (Lunt *et al.*, 1998; Taberlet *et al.*, 1998; Hewitt, 1999; Petit *et al.*, 2002; Magri *et al.*, 2006). Furthermore, plastid haplotype diversity is high in the south-eastern populations (populations 14–22) and the most distinct haplotype XI is restricted to marginal populations in the south-east (Fig. 3f–h) in line with the rear edge hypothesis (Hampe & Petit, 2005; Grassi *et al.*, 2009). In contrast to *E. serpyllifolius*, in *E. tenuifolius* the deepest phylogeographic splits inferred from AFLP and cpDNA sequence data are at different locations (Fig. 3e–h). This suggests that the precise location of refugia and/or the extent of range expansion differed among cycles of cooler and warmer periods, which is highly plausible for a species with latitudinal range shifts in an area without obvious dispersal barriers.

The displacement refugia model, formulated for co-distributed, but elevationally differentiated mountain taxa, predicts that high-elevation species faced range fragmentation mostly in the warmer but short interglacial periods, whereas range fragmentation for low-elevation species occurred mostly during the cold and long glacial periods (Kropf *et al.*, 2003). Consequently, low-elevation species are expected to show higher genetic differentiation and stronger phylogeographic signal than high-elevation species, as demonstrated in the southern European (mostly) non-alpine *Anthyllis montana* and the alpine *Pritzelago alpina* by Kropf *et al.* (2002, 2003). Evaluating these predictions on the high-elevation *E. serpyllifolius* and the low-elevation *E. tenuifolius*, which not only have overlapping distribution ranges (Fig. 1), but also share pollination and dispersal syndromes, identified no significant difference between the two species (mean rescaled net number of nucleotide differences: 0.110 ± 0.050, 0.107 ± 0.042; $P = 0.36$). As the initial study included two unrelated species, which are latitudinally differentiated (temperate *Pritzelago* vs. submediterranean *Anthyllis*; Meusel *et al.*, 1965), the observed differences between these two taxa might be due to species-specific features, such as different pollination or dispersal biology, rather than elevational differentiation. Further data on sympatric but elevationally differentiated taxa with comparable biological features will be necessary to assess whether a general pattern, as implied by the displacement refugia model, exists.

CONCLUSIONS

We have shown that two species that have similar biological attributes and overlapping distributions, but that are elevationally segregated, differ in their responses to Pleistocene climatic fluctuations. Our results, however, indicate that the observed genetic patterns discourage simplistic models of responses to Pleistocene climatic fluctuations and favour more complex scenarios for individual species. Our results show that mostly elevationally migrating mountain species may show moderate levels of gene flow between isolated mountain ranges, while lowland species experiencing mostly latitudinal range shifts may show considerable phylogeographic structure due to restriction to several isolated refugia. Further studies in the western Balkan refugium will be necessary to test whether the phylogeographic histories of the *Edraianthus* species investigated are idiosyncratic or represent more general patterns.

ACKNOWLEDGEMENTS

We thank Darko Mihelj, Martin Turjak and Božo Frajman for collecting some of the plant samples. Chris Dixon and Michael Barfuss helped in the lab, and Božo Frajman commented on previous versions of the manuscript. Funding by the European Commission (FP6 Marie Curie Intra-European Fellowship grant EVOBALK to B.S.) is gratefully acknowledged.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Appendix S1 Analysis of the amplified fragment length polymorphism (AFLP) data sets with STRUCTURE 2.2.

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Editor: Jens-Christian Svenning