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ORIGINAL ARTICLE

Molecular study of the *Cardamine maritima* group (Brassicaceae) from the Balkan and Apennine Peninsulas based on amplified fragment length polymorphism

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Abstract The amphi-Adriatic region, and especially the Western Balkan Peninsula, belongs to the most important biodiversity hotspots in the temperate region. Nevertheless, detailed phylogeographic and molecular systematic studies in the Western Balkan are rare due to sporadic sampling in regions, where access has been, until recently, restricted by war. The *Cardamine maritima* group, which is the focus of this study, comprises not only the currently recognised species *C. maritima* and *C. monteluccii*, but also other taxa, which have been rendered to synonymy by most of the national floras and checklists. Molecular data acquired by the amplified fragment length polymorphism method showed a clear pattern within the group. Italian populations of *C. monteluccii* are well separated from Balkan taxa. In a step forward from previous taxonomic confusion surrounding Balkan populations, the present study confirms that five allopatric units—each with a clearly delimited and a rather restricted distribution range—can be easily recognised here. They correspond to *C. fialae*, *C. serbica*, *C. rupestris*, and two genetically distant and allopatric units within *C. maritima*. While individual taxa gained high bootstrap support in the neighbour-joining tree, there is low support for the internal nodes and it is hard to infer any relationships among taxa based on this information. The majority of Balkan populations of the *C. maritima* group exhibit features of genetic variability that enable us to hypothesise that these populations are relic ones.

Keywords AFLP · Apennine Peninsula · Balkan Peninsula · Glacial refugia · Genetic diversity

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Introduction

The Balkan Peninsula, with its environmental stability and topographic and climatic diversity, is a fascinating region for phylogenetic and phylogeographic studies, because it harbours high floral and faunal diversity (Turrill 1929; Polunin 1997; Kryštufek and Reed 2004), served as a refugium during the Pleistocene (Comes and Kadereit 1998; Hewitt 2000; Hampe et al. 2003; Petit et al. 2003; Eastwood 2004; Tzedakis 2004; Médail and Diadema 2008), and has received very little attention until now. Examples of a few previous studies include examinations of genetic diversity within and between species of Balkan beech populations (Gömöry et al. 1999) using isozymes; the Balkan-endemic Martino's vole (Kryštufek et al. 2007), isophyllous species of *Campanula* (Park et al. 2006) and the genus *Heliosperma* (Frajman and Oxelman 2007) all by employing DNA sequence data.

Climatic fluctuations of the late Pliocene and Pleistocene and subsequent changes in Adriatic sea level led to the formation of landbridges in the northern part of the sea (Colantoni et al. 1979; Voges 1995), enabling trans-Adriatic exchange, while in warmer periods the northern Adriatic basin was flooded and acted as a barrier, supporting vicariance events in a previously continuous distribution area. Park et al. (2006) hypothesised that repeated cycles of isolation of populations during climatically more favourable periods reinforced speciation of *Campanula* taxa on the east and west Adriatic coasts. Similarly, isolation of populations on a small geographical scale during interglacial periods, with secondary contacts during glacial maxima, shaped the present patterns of genetic variation in Martino's vole (*Dinaromys bogdanovi*, Kryštufek et al. 2007) and the *Heliosperma pusillum* group (Frajman and Oxelman 2007) within the western Balkan Peninsula. It can be expected that similar evolutionary patterns exist in other species.

The amphi-Adriatic region, and especially the Western Balkan Peninsula, is important for better understanding the diversity and history of the Mediterranean flora. Detailed studies of genetic variation of taxa in this region are also important for establishing conservation priorities. Nevertheless, such small-scale phylogeographic and other molecular studies in the Western Balkan are rare due to sporadic sampling in regions where access has been, until recently, restricted by war (but see Park et al. 2006; Kryštufek et al. 2007; Frajman and Oxelman 2007).

The genus *Cardamine* is represented in Europe by approximately 54 species (Lihová and Marhold 2006). Our current study concentrates on the least-known European species group of the genus, which comprises the Western Balkan *C. maritima* DC. (Candolle 1821) (including *C. fialae* Fritsch) and the Apennine *C. monteluccii* Br.-Cat. & Gubell. (Brilli-Cattarini and Gubellini 1986) in the circumscription as given in the Flora Europaea (Jones and Akeroyd 1993) and Atlas Florae Europaeae (Jalas and Suominen 1994). Although only these two species are currently generally accepted, another four taxa from the Western Balkan Peninsula were described as being part of this group, namely *C. fialae* Fritsch (Fritsch 1897), *C. maritima* var. *maglicensis* Rohlena (Rohlena 1906), *C. serbica* Pančić (Pančić 1884), and *C. maritima* "proles" *rupestris* O. E. Schulz (Schulz 1903). Jones and Akeroyd (1993) and Jalas and Suominen (1994) doubted the status of *C. fialae*, considering it to be "perhaps a subspecies of *C. maritima.*"

Data from Brilli-Cattarini and Gubellini (1986) and our preliminary results (J. Kučera et al., unpublished data) have shown that the taxa of this group are diploid. Populations included here are annual or biennial, and they occupy calcareous rocks and sliderocks. Both autogamy and allogamy were proven in a preliminary study of the breeding systems of *C. maritima*, *C. monteluccii*, and *C. rupestris* (J. Kučera, unpublished data).

Results of the study of hexaploid *C. asarifolia* L. by Lihová et al. (2006), which was based on ITS and CHS sequences and which also included some taxa treated here as *C. maritima*, *C. rupestris* (O.E. Schulz) K. Maly, *C. fialae*, *C. serbica*, and *C. monteluccii*, indicate that the *C. maritima* group is monophyletic. The cpDNA sequences (*trn*L-*trn*F), however, are not as conclusive (Lihová et al. 2006). This study also indicated this group's basal position among European *Cardamine* diploids, as well as its considerable genetic variation.

More than a century ago, Fritsch (1895) and also Schulz (1903) hypothesised that *C. maritima* is a hybrid species, which arose from the hybridisation of *C. glauca* DC. and *C. graeca* L. According to Schulz (1903, p. 580), *C. maritima* "proles" *rupestris* is morphologically closer to *C. glauca*, while *C. maritima* "proles" *serbica* (Pančić) O. E. Schulz more closely resembles *C. graeca*. *C. rupestris* is sometimes considered to be a hybrid between *C. glauca* and *C. maritima* (Beck 1903; Hayek 1927; Rohlena 1942). Indeed, both *C. glauca* and *C. graeca*—like *C. maritima*—were shown to occupy a basal position with respect to other European diploid *Cardamine* taxa (Lihová et al. 2006).

The distribution areas of *Cardamine maritima* and related taxa on the Western Balkan Peninsula largely coincide with major refugia of Mediterranean plants during glacial periods (Médail and Diadema 2008). Refugial populations usually harbour higher allelic diversity compared with those that experienced serious bottlenecks (Widmer and Lexer 2001). It is therefore expected that the genetic variation and consequently also the taxonomic structure of the group of *C. maritima* may differ from those of other taxa of the genus *Cardamine* distributed in the areas seriously influenced by Pleistocene glaciations (Lihová et al. 2003; Kučera et al. 2006; Perný et al. 2005a, b, c).

The main questions addressed by this study are as follows: (1) What are the patterns of genetic variation among populations of *C. monteluccii*, broadly conceived *C. maritima*, and the related taxa *C. glauca* and *C. graeca*? (2) Is there any support for the currently recognised or previously described taxa; should they be taxonomically recognised? (3) Is there any genetic evidence among the studied taxa, supporting small-scale (mainly altitudinal) distribution shifts influenced by glaciation events as opposed to the large-scale latitudinal shifts? In contrast to species in northern European areas, species in southern European regions survived Pleistocene glaciations without large geographical displacements, but rather by ascending or descending mountains, as exemplified in *Armeria* (Gutiérrez Larena et al. 2002). The hypothesised long-term in situ survival in

mountains of the Balkan Peninsula without the need for large-scale geographical migrations and gene flow should result in isolated and highly divergent genetic entities, which should coincide with mountain ranges. A high number of fragments should be found exclusively in separated mountain ranges.

Amplified fragment length polymorphism (AFLP, Vos et al. 1995) is a standard marker for assessing inter- (Prohens et al. 2006; Guo et al. 2005; Nguyen et al. 2004) and intraspecific (Pilon et al. 2007; Marghali et al. 2005; Juan et al. 2004) genetic variation and differentiation. Despite some concerns that AFLP fragments of the same length seen in two species might not be homologous and/or some fragments might not be independent, it was suggested that this method can be used for phylogenetic reconstructions, especially for species groups that have diverged recently (e.g. Bussell et al. 2005; Koopman 2005). Although a dominant marker, AFLP is powerful in addressing questions like those in our study (e.g. Prohens et al. 2006; Lara-Cabrera and Spooner 2004) and was also successfully applied to other *Cardamine* species complexes—both diploid and polyploid (Lihová et al. 2003, 2004a, b; Perný et al. 2005a, b).

Materials and methods

Plant material

Plant material was collected with the aim of getting representative samples of *C. maritima* and related taxa from both the Apennine and Balkan Peninsulas. Material includes samples from populations previously classified as *C. maritima*, *C. rupestris*, *C. monteluccii*, *C. fialae*, *C. maritima* var. *maglicensis*, and *C. serbica*, including type localities of the latter three taxa. In addition, samples from two populations of *C. graeca* (from the Apennine and Balkan Peninsulas) and from five populations of *C. glauca* were included, as these taxa were hypothesised to be parental species of *C. maritima* and, like this species, were shown to be in a basal position with respect to other European diploid *Cardamine* taxa (Lihová et al. 2006). Samples included silica-gel-dried material from 1 to 5 individual plants per population. Details of the localities are given in Table 1, and their locations are shown in Fig. 1a, b.

Amplified fragment length polymorphism

For extracting genomic DNA, we used silica-gel-dried leaves. DNA was extracted using CTAB isolation buffer (Doyle and Doyle 1987). Isolated genomic DNA was checked on an agarose gel and quantified with a UV 160A Spectrophotometer. The AFLP procedure was performed according to a standard protocol (Applied Biosystems 1996) with minor modifications. The genomic DNA was cut with *Mse*I and *Eco*RI restriction endonucleases and ligated with two double *Mse*I and *Eco*RI adaptors for 2 h at 37°C in a thermal cycler (GeneAmp® PCR System 9700, PE Applied Biosystems). The product was diluted with $TE_{0.1}$ buffer. For pre-selective amplification in a thermal cycler, the following program was used: an initial hold at 72°C for 2 min; 20 cycles of 94°C for 1 s, 56°C for 30 s, and 72°C for 2 min; a final hold at 60°C for 30 min; and cooling to 4°C. The product of pre-selective amplification was checked on an agarose gel and diluted with $TE_{0.1}$ buffer. For selective amplification, *Eco*RI-AAG (HEX)/*Mse*I-CTG, *Eco*RI-ATC (6-FAM)/*Mse*I-CAG, and *Eco*RI-AGC (NED)/*Mse*I-CTG selective primer combinations (VBC Genomics, Vienna) were used, together with the following selective amplification program in a thermal cycler: an initial cycle of 94°C for 2 min, 65°C for 30 s, and 72°C for 2 min; eight cycles of 94°C for 1 s, 64°C for 30 s (decreasing by 1°C in each cycle from 64°C to 57°C), and 72°C for 2 min; 23 cycles of 94°C for 1 s, 56°C for 30 s, and 72°C for 2 min; a final hold at 60°C for 30 min; and cooling to 4°C. The products were loaded on 5% polyacrylamide gels with the size standard GeneScan 500 ROX on an automated sequencer (ABI 377, PE Applied Biosystems). AFLP fragments were analysed by GeneScan® (PE Applied Biosystems) and the Genographer program (version 1.6.0, ©Montana State University, 1999; http://hordeum.msu.montana.edu/genographer/).

Data analysis

Koopman (2005) tested the congruence of AFLP and ITS topologies; through the study of the example genus *Lactuca* s.l. as well as based on data from the literature survey of the wider spectrum of species and genera, he demonstrated that AFLP-based relationships among genotypes in plants that are 10–30 (or possibly up to 35) ITS-nucleotides apart are usually recovered with good bootstrap support. Therefore, AFLP markers in plants are likely to be phylogenetically informative at this level of ITS sequence divergence. The amount of sequence divergence among plants of the *C. maritima* group ascertained by Lihová et al. (2006) in the internal transcribed spacer region (ITS) was up to 21 bp, while the divergence was up to 54 bp when plants of the *C. maritima* group, *C. graeca*, and *C. glauca* were included. This indicates that, at least for the evaluation of the relationships within the *C. maritima* group, AFLP data represent an adequate tool. To avoid potential bias caused by the inclusion of *C. graeca* and *C. glauca*, some analyses were performed both with and without these taxa.

Table 1 Origin of plant material used for AFLP analyses

Taxon/code, collection data	AFLP
Cardamine monteluccii	
Tuscany 1—Italy, Tuscany, Roccalbegna, loc. Pescinello, 42°47′41″N, 11°31′17″E, 740 m a.s.l., 8 May 2003, *JK*, *MK and FS*	2
Tuscany 2—Italy, Tuscany, Semproniano, settlement of Rocchete, 42°44′27″N, 11°30′07″E, 505 m a.s.l., 8 May 2003, *JK and MK*	2
Marche 1—Italy, Marche, near the town of Pióraco, 43°10′00″N, 12°58′36″E, 475 m a.s.l., 9 May 2003, *JK and MK*	3
Marche 2—Italy, Marche, Mt. San Vicino, Valle dell Aquarella, 43°19′55″N, 13°01′21″E, 682 m a.s.l., 9 May 2003, *JK and MK*	3
Cardamine maritima	
Velebit 2—Croatia, Velebit Mts., Tribanj, near the settlement of Poljak, Tribanjska draga gorge, 44°30′49″N, 15°06′03″E, 11 m a.s.l., 18 April 2003, *JK and MK*	3
Velebit 1—Croatia, Velebit Mts., Starigrad, village of Paklenica, Velika Paklenica gorge, 18 April 2003, *JK and MK*	5
Velebit 3—Croatia, Velebit Mts., Starigrad, village of Seline, Mala Paklenica gorge, 44°16′59″N, 15°29′34″E, ca. 250 m a.s.l., 19 April 2003, *JK and MK*	2
Mosor 1—Croatia, Mosor Mts., Split, near the village of Gornje Sitno, 43°31′12″N, 16°36′11″E, 649 m a.s.l., 21 April 2003, *JK and MK*	4
Biokovo 1—Croatia, Biokovo Mts., Baška voda, village of Bast, 43°21′31″N, 16°59′18″E, 398 m a.s.l., 24 April 2003, *JK and MK*	4
Velebit 4—Croatia, Velebit Mts., Karlobag, between the villages of Vidovac and Sušanj, 44°30′38″N, 15°07′13″E, 436 m a.s.l., 25 April 2003, *JK and MK*	4
Risan—Montenegro, above the town of Risan, 42°31′57″N, 18°42′02″E, 504 m a.s.l., 22 April 2003, 4 May 2004, *JK and MK*	4
Ledeniće—Montenegro, Risan, village of Ledeniće, 42°32′42″N, 18°42′45″E, 695 m a.s.l., 22 April 2003, *JK and MK*	4
Lovćen 1—Montenegro, Mt. Lovćen, Kotor, near the road from Kotor to village of Krstac, 42°24′24″N, 18°46′36″E, 423 m a.s.l., 22 April 2003, *JK and MK*	4
Lovćen 2—Montenegro, Mt. Lovćen, Kotor, village of Njegoši, 42°24′38″, 18°47′23″, 919 m a.s.l., 4 May 2004, *JK and MK*	3
Cardamine fialae	
Ružići—Bosnia and Herzegovina, Grude, near the village of Ružići, 43°19′10″N, 17°26′09″E, 287 m a.s.l., 23 April 2003, *JK and MK*	4
Klobuk—Bosnia and Herzegovina, Ljubuški, near the village of Klobuk, 43°18′43″N, 17°26′00″E, 285 m a.s.l., 23 April 2003, *JK and MK*	4
Grude—Bosnia and Herzegovina, near the town of Grude, 43°20′38″N, 17°25′45″E, 298 m a.s.l., 23 April 2003, *JK and MK*	5
Cardamine serbica (“*Cardamine maritima* var. *maglicensis*”)	
Maglić 1—Montenegro, Maglić Mts., 8 km N of the town of Plužine, 43°12′37″N, 18°51′33″E, 700 m a.s.l., 15 July 2003, *JK and MK*	5
Maglić 2—Montenegro, Maglić Mts., Plužine, village of Mratinje, 43°16′07″N, 18°50′05″E, 708 m a.s.l., 15 July 2003, *JK and MK*	5
Maglić 3—Montenegro, Maglić Mts., town of Plužine, 43°10′12″N, 18°51′31″E, 708 m, 5 May 2004, *JK and MK*	2
Cardamine serbica	
Tara—Serbia, Tara Mts., Bajina Bašta, village of Perućac, 43°57′38″N, 19°22′53″E, 285 m, 22 May 2004, *JK and MK*	3
Cardamine rupestris	
Čevo 1—Montenegro, Cetinje, village of Čevo, 42°31′23″N, 18°55′04″E, 817 m, 4 May 2004, *JK and MK*	2
Čevo 2—Montenegro, Cetinje, near the village of Čevo, 42°33′00″N, 18°54′12″E, 643 m, 4 May 2004, *JK and MK*	4
Grahovo—Montenegro, Nikšić, village of Grahovo, 42°38′35″N, 18°41′02″E, 689 m, 5 May 2004, *JK and MK*	5
Cardamine graeca	
Grude—Bosnia and Herzegovina, near the town of Grude, 43°20′38″N, 17°25′45″E, 298 m a.s.l., 23 April 2003, *JK and MK*	5
Marche—Italy, Marche, near the town of Pióraco, 43°10′00″N, 12°58′36″E, 475 m a.s.l., 9 May 2003, *JK and MK*	4
Cardamine glauca	
Risan—Montenegro, above the town of Risan, 42°31′57″N, 18°42′02″E, 504 m a.s.l., 4 May 2004, *JK and MK*	3
Lovćen—Montenegro, Mt. Lovćen, Kotor, near the road from Kotor to village of Krstac, 42°26′13″N, 18°51′11″E, 1,058 m a.s.l., 4 May 2004, *JK and MK*	3
Maglić—Montenegro, Maglić Mts., Plužine, village of Mratinje, 43°16′07″N, 18°50′05″E, 708 m a.s.l., 15 July 2003, 5 May 2004, *JK and MK*	3
Spila—Montenegro, Nikšić, village of Spila, 42°44′41″N, 18°41′43″E, 841 m a.s.l., 5 May 2004, *JK and MK*	3
Ledeniće—Montenegro, Risan, village of Ledeniće, 42°32′42″N, 18°42′45″E, 695 m a.s.l., 4 May 2004, *JK and MK*	1
Cetinje—Montenegro, near the town of Cetinje, 42°23′54″N, 18°53′08″E, 819 m a.s.l., 4 May 2004, *JK and MK*	2

Collectors: *JK* J. Kučera, *MK* M. Kolník, *FS* F. Selvi

AFLP number of plants used in the AFLP analyses

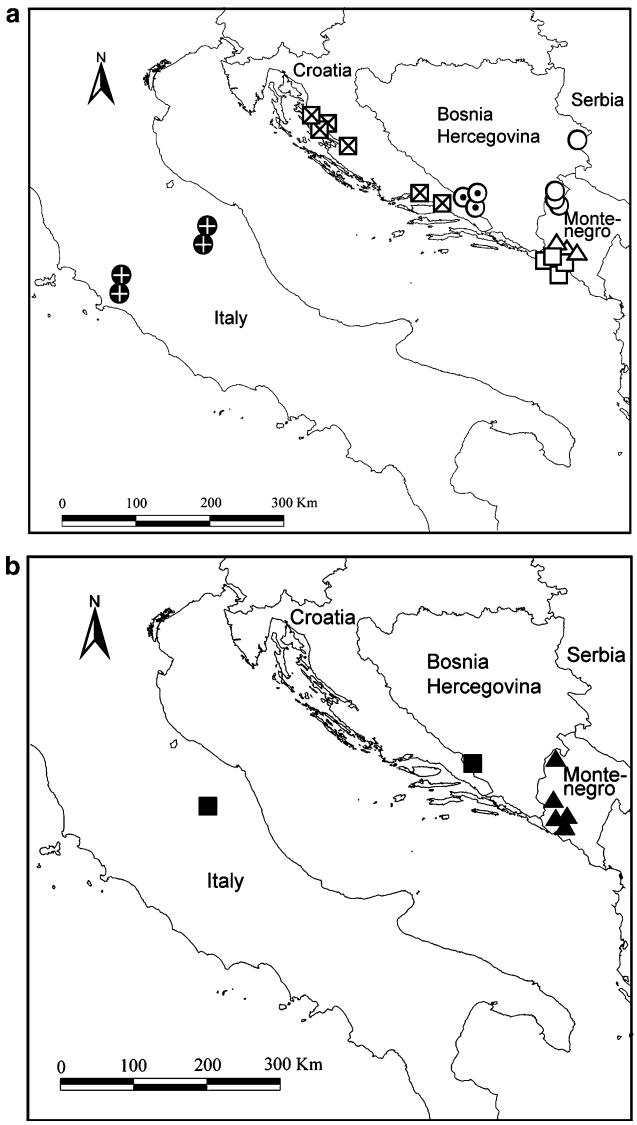

Fig. 1 **a** Map showing the distribution of the sampled material of *C. monteluccii* (*circle with cross*), *C. maritima* populations from Croatia (*square with cross*), *C. maritima* populations from Montenegro (*square*), *C. fialae* (*circle with dot*), *C. rupestris* (*triangle*), and *C. serbica* (*circle*). **b** Map showing the distribution of the sampled material of *C. graeca* (*square*) and *C. glauca* (*triangle*)

The presence or absence of fragments ranging from 75 to 500 bp was scored for each sample and transferred into a binary matrix. The secondary matrix was computed with Jaccard's coefficient, and principal coordinate analysis (PCoA) was subsequently performed with the SYN-TAX 2000 package (Podani 2001). The neighbour-joining tree was computed using Nei and Li (1979) genetic distance in the PAUP* program (version 4.0b10, Swofford 2003). The tree was rooted at the midpoint. Group support was assessed with the same program by repeated bootstrap analyses with 5,000 replications.

To estimate the pattern of variation of the material studied by clustering individuals into groups, Bayesian analysis of "population" structure was carried out using the program BAPS 3.2 (Corander et al. 2006). In this analysis, both the frequencies of AFLP fragments and the number of genetically diverged groups are treated as random variables. Stochastic optimisation is used to infer the posterior mode of the genetic structure. It searches for the number of clusters with the highest natural logarithm of the marginal likelihood of the data and gives a clustering of individuals for the best solution.

For each previously recognised taxon or grouping resulting from the Bayesian analysis of population structure, as well as for each population, the following parameters were calculated: total number of fragments (bands) per taxon or population; average, minimum, and maximum number of fragments per individual; and number of exclusive fragments (present in a given taxon only, but not necessarily in all its samples). In addition, for each previously recognised taxon or grouping resulting from the Bayesian analysis of population structure, the proportion of polymorphic fragments was calculated.

To avoid subjective definitions of rare markers [e.g., markers present in <10% of the investigated individuals (Stehlik et al. 2002) or in less than a certain number of individuals (Tribsch et al. 2002)], we followed Schönswetter and Tribsch (2005) in calculating "frequency-downweighted marker values" (DW) equivalent to range-downweighted species values in historical biogeographical research (Crisp et al. 2001). These values were calculated for each previously recognised taxon or grouping resulting from the Bayesian analysis of population structure, as well as for each population.

DW, the number of fragments, the number of exclusive fragments, and the proportion of polymorphic fragments depend on the number of sampled individuals in each group (population or species). Usually, when the groups are represented by different numbers of individuals in the dataset, individuals are randomly deleted to obtain equal numbers of individuals (e.g. by Schönswetter and Tribsch 2005). However, if several plants are deleted from the sample, much of the information is wasted by this approach, and there is also a greater danger of obtaining incorrect results just by chance. Therefore, we repeated the resampling of the whole dataset using our own scripts with Scilab software (http://www.scilab.org) to achieve the same sample size in each taxon/group or population. Replications were allowed in the resampling (i.e. one individual may occur more than one time in the resulting dataset). Hence, our approach is a special case of bootstrap analysis, and it is possible to estimate sample characteristics based on the repeated resampling. We selected nine individuals per taxon or group as defined by NJ and Bayesian clustering in each step and two individuals per population in each step. (The resampling has been done separately for taxa and populations.)

Thousand replications were used for calculating the number of fragments and the proportion of polymorphic fragments, whereas 10,000 replications were used for calculating DW and the number of exclusive fragments, because these characteristics are influenced not only by the selection of individuals within the respective group but also by the selection of individuals within the other groups. Minimum and maximum or average values based on corresponding resamplings are presented.

Additional genetic diversity parameters were calculated using POPGENE 1.32 (Yeh et al. 1997). For each taxon (or group of populations from a particular area) and population, the following parameters were calculated: H_{Nei}—Nei's average gene diversity corrected for small sample size (Nei 1978; see also Bonin et al. 2007: Box 2; Kosman 2003) and Shannon's diversity index (Lewontin 1972). AFLP fragment sharing among particular pairs or groups of taxa and populations was evaluated as well.

Differentiation between populations and the geographic structure of genetic variation was studied by analysis of molecular variance (AMOVA). Original binary matrices were divided into separate matrices for each taxon. Molecular variance was calculated from a matrix of squared Euclidean distances using the program ARLEQUIN (version 2.000, Schneider et al. 2000). Total genetic variation was partitioned into levels: among individuals within populations, among populations, and among groups or regions. Thousand permutations were run to obtain test statistics.

Results

NJ tree

A mid-point-rooted neighbour-joining tree (Fig. 2) of the *C. maritima* group (without samples of *C. glauca* and *C. graeca*) shows *C. monteluccii*, *C. fialae*, and *C. rupestris* in clusters with 99 or 100% bootstrap support. *Cardamine maritima* from Croatia and from Montenegro form clusters with 96 and 88% support, respectively. These clusters appear on different parts of the tree. *Cardamine serbica* and *C. maritima* var. *maglicensis* appear in one cluster with 100% support, with *C. serbica* nested within *C. maritima* var. *maglicensis*. Other internal nodes on the tree have only low support, except for the cluster containing *C. serbica* and *C. maritima* var. *maglicensis* (from here on referred to as *C. serbica* + *C.* maglicensis*), and *C. rupestris*, which has 84% support. When *C. glauca* and *C. graeca* are included in the tree (Fig. 3), all the abovementioned clades have 86–100% support, but there is no support for the clade *C. serbica* + *C.* maglicensis* and *C. rupestris*. Both *C. graeca* and *C. glauca* have 100% support.

Principal coordinates analysis

On the ordination diagram of the principal coordinates analysis (PCoA) based on AFLP data (Fig. 4), individuals of most of the previously recognised taxa formed their own groupings, which clearly differentiated from other taxa. Similar to the NJ tree, the sole exceptions were the division of *C. maritima* into two isolated groupings, reflecting their geographical isolation (Croatia vs. Montenegro), and *C. maritima* var. *maglicensis* and *C. serbica*, which formed one grouping. Samples were divided mainly along the first principal coordinate, where *C. fialae*, *C. maritima* from Croatia and Montenegro, and *C. monteluccii* appear on the left side of the diagram, while *C. glauca*, *C. graeca*, *C. serbica* + *C.* maglicensis*, and *C. rupestris* are on the right side of the diagram. *Cardamine glauca* and *C. serbica* + *C.* maglicensis* appear on extreme ends of the second principal coordinate.

BAPS

The optimal partition with the highest log marginal likelihood (−5,417.9) produced by BAPS consisted of eight clusters that corresponded to the groups in the NJ and PCoA analyses (Figs. 2, 3, 4).

AFLP fragments and their sharing

The AFLP analysis of *C. maritima* and related taxa, 110 plant individuals in total, resulted in 232 scorable fragments or bands. Fourteen fragments were distributed to a single individual and one fragment was monomorphic. (The number of monomorphic fragments increased to six when *C. glauca* and *C. graeca* were not included in the analysis.) The average total number of fragments per species was highest for *C. maritima* from Croatia and from Montenegro (68.80 and 50.95 fragments) and lowest for *C. graeca*, *C. serbica* + *C.* maglicensis*, and *C. rupestris* (27.97, 33.92, and 34.51 fragments, respectively; Table 2). The average number of fragments per individual was higher in populations of *C. maritima* from Montenegro and Croatia, *C. fialae*, and *C. monteluccii*, ranging from 36.12 to 41.17 fragments. Lower values were found in *C. graeca* (24.43), *C. glauca* (26.34), *C. rupestris* (28.36), and *C. serbica* + *C.* maglicensis* (29.13).

The highest number of exclusive fragments per species (or group of populations) was observed in *C. glauca* (33.10) and *C. maritima* from Croatia (26.30). The lowest number was found in *C. fialae* (13.54). When the number of exclusive fragments was calculated for populations, a tendency toward higher values was found in *C. glauca*, *C. graeca*, both entities of *C. maritima*, and *C. fialae*.

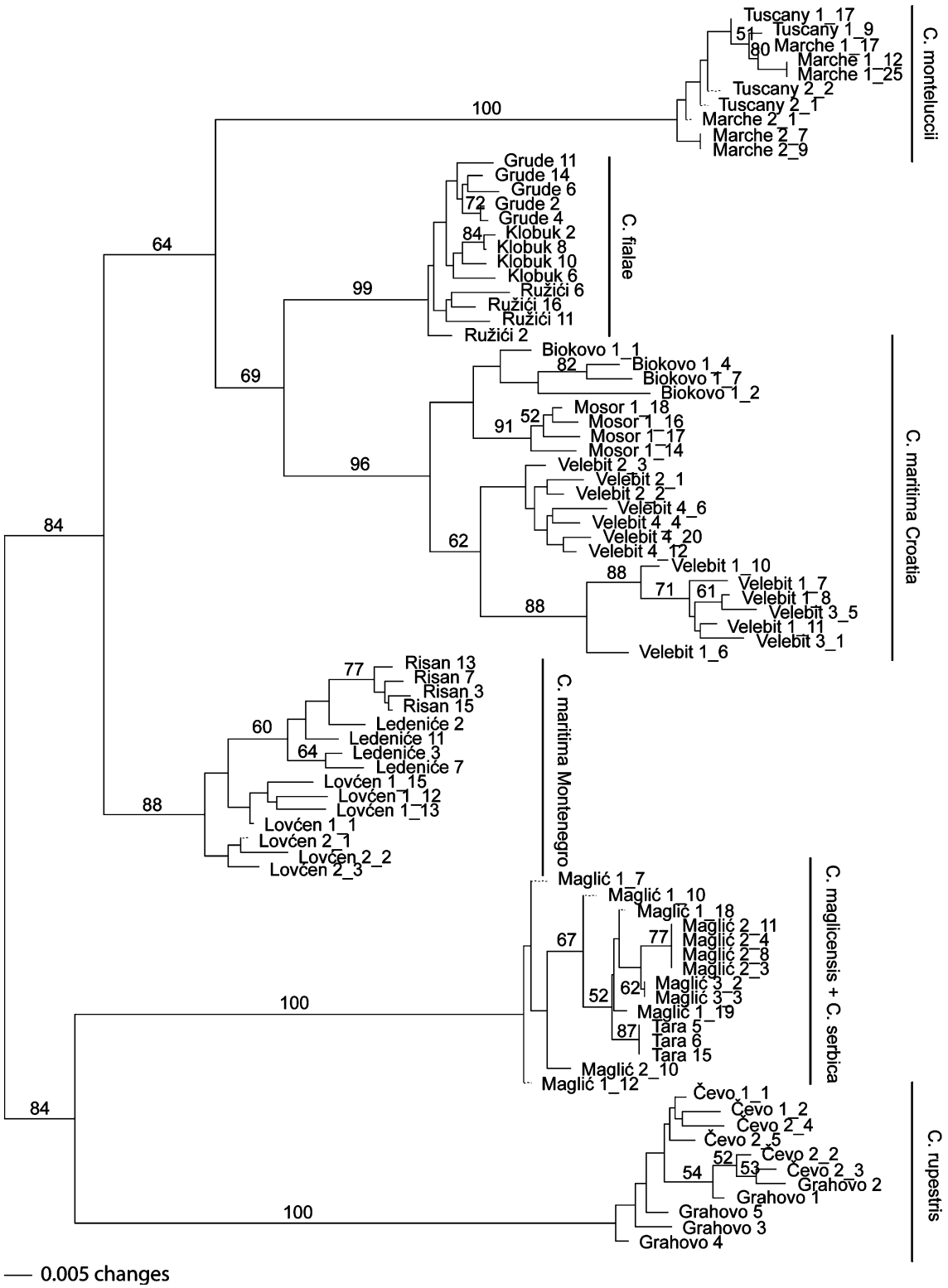

Fig. 2 Neighbour-joining analysis of AFLP data of the *Cardamine maritima* group. Bootstrap support ≥50% is shown above branches. For population codes, see Table 1

Lower values were found in *C. rupestris* and *C. serbica* + *C.* maglicensis*; no exclusive fragments were found for populations of *C. monteluccii*.

The frequency-downweighted marker values (DW) calculated for species were high in *C. maritima* from Croatia and in *C. glauca* (38.01 and 35.50); intermediate for

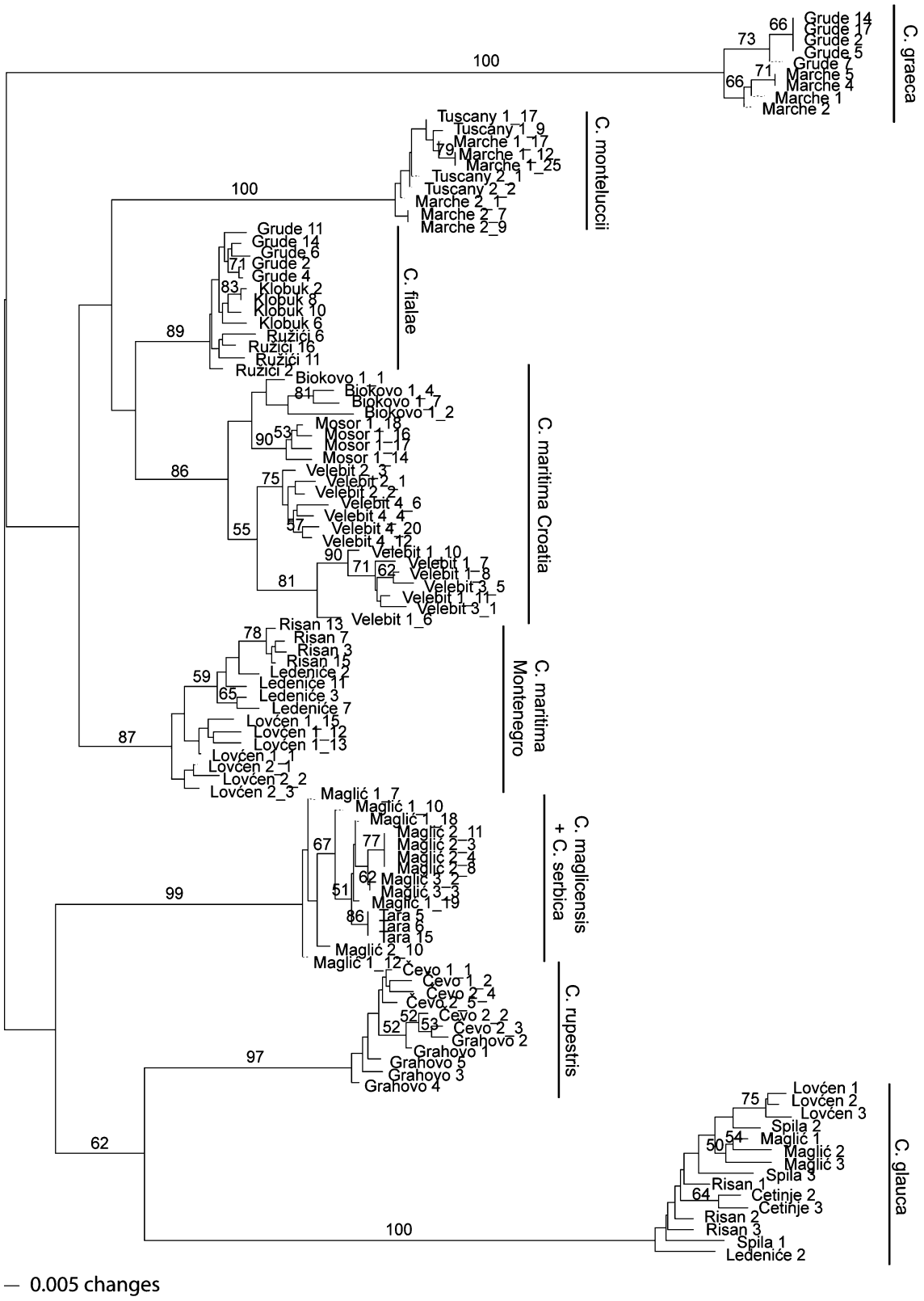

Fig. 3 Neighbour-joining analysis of AFLP data of the *Cardamine maritima* group, *C. glauca*, and *C. graeca*. Bootstrap support ≥50% is shown above branches. For population codes, see Table 1

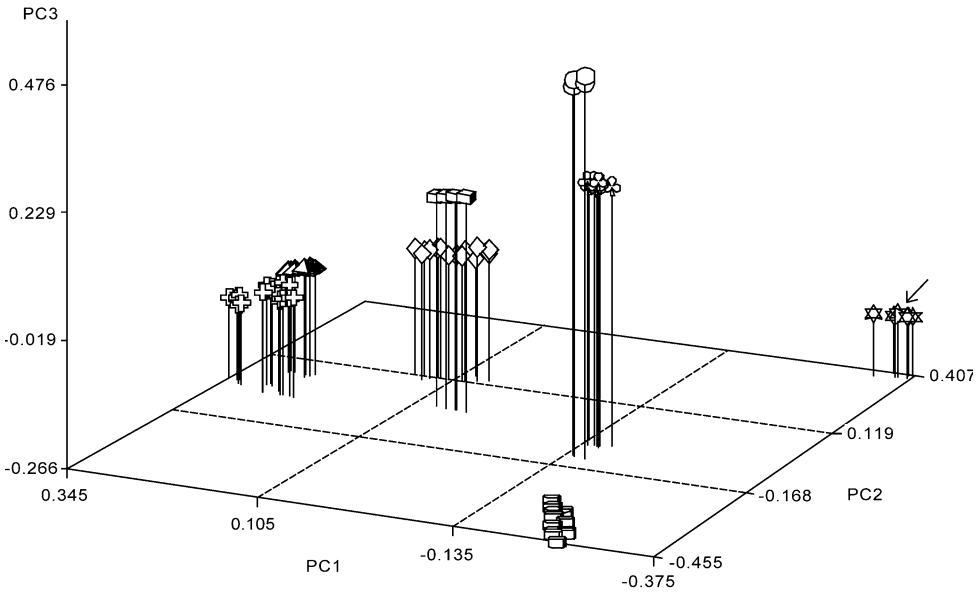

Fig. 4 Principal coordinate analysis of AFLP data (based on Jaccard's coefficient) of *C. graeca* (*balloon*), *C. monteluccii* (*cube*), *C. rupestris* (*club*), *C. fialae* (*pyramid*), *C. maglicensis* (*star*), *C. serbica* (*flag*, marked by arrow), *C. maritima*—Montenegro (*diamond*), *C. maritima*—Croatia (*cross*), and *C. glauca* (*cylinder*). The first three coordinates explain 15.22, 12.99, and 10.16% of the total variation, respectively

Table 2 Distribution of AFLP fragments and genetic diversity measures in the species of the *C. maritima* group (including two entities in *C. maritima*), *C. glauca*, and *C. graeca*

Taxon	*n* (n_{pop})	FT	FA	*F* (min–max)	EF	DW	%Pop	H_{Nei}	H_{Sh}
C. monteluccii	10 (4)	42.98	40.07	**37.78–42.44**	17.82	25.59	*13.76*	*0.0123*	*0.0129*
C. fialae	13 (3)	49.77	39.99	**38.56–42.00**	*13.54*	25.64	35.93	0.0300	0.0312
C. serbica + *C.* maglicensis*	15 (4)	33.92	29.13	27.78–30.22	*16.70*	*21.01*	*20.43*	*0.0123*	*0.0127*
C. rupestris	11 (3)	34.51	28.36	26.89–29.33	*16.11*	*21.54*	35.60	0.0240	0.0251
C. maritima—Montenegro	15 (3)	50.95	36.12	**34.00–38.56**	*15.78*	26.50	**49.99**	**0.0467**	**0.0483**
C. maritima—Croatia	22 (5)	68.80	41.17	**37.11–44.67**	**26.30**	**38.01**	**68.72**	**0.0845**	**0.0865**
C. glauca	15 (6)	42.08	26.34	*25.11–27.33*	**33.10**	**35.50**	**61.49**	**0.0434**	**0.0450**
C. graeca	9 (2)	27.97	24.43	*23.56–25.33*	19.83	*22.53*	*24.79*	*0.0146*	*0.0155*

Repeated resamplings were made to achieve the same sample size in each group (for details, see "Material and methods")

For *F* (min–max), EF, DW, %Pop, H_{Nei}, and H_{Sh}, high values are in bold, intermediate values in normal type, and low values in italics

C. maglicensis* = *C. maritima* var. *maglicensis*

n total number of individuals; n_{pop} number of populations; *FT (fragments total)* average total number of fragments per taxon; *FA (fragments average)* average number of fragments per individual; *F (min–max)* minimum and maximum number of fragments per individual; *EF (exclusive fragments)* average number of fragments present in a given taxon, but absent in other taxa; *DW* average frequency-downweighted marker values; *%Pop* average percentage of polymorphic fragments in a given taxon (or group of populations); H_{Nei} Nei's gene diversity; H_{Sh} Shannon's diversity index

C. maritima from Montenegro, *C. fialae*, and *C. monteluccii* (26.50, 25.64, and 25.59, respectively); and low for *C. graeca*, *C. rupestris*, and *C. serbica* + *C.* maglicensis* (22.53, 21.54, and 21.01, respectively; Table 2). When DW values were calculated for populations, there were no conspicuous differences among taxa, except for higher values for *C. graeca* (Table 3).

With respect to the shared fragments calculated from the whole data set (without resampling), the least similar taxa were *C. glauca*, *C. graeca*, *C. rupestris*, and *C. serbica* + *C.* maglicensis*; they shared only one or two exclusive fragments with other taxa. The remaining taxa shared more fragments. *Cardamine maritima* from Croatia, the taxon sharing the most fragments with other taxa, shared five exclusive fragments with *C. fialae*, three with *C. monteluccii*, and four with *C. maritima* from Montenegro.

Analyses of molecular variance and genetic diversity

The highest genetic variation appeared among groups that resulted from the NJ and BAPS analyses. In such cases, the variation among groups was as high as 77.04% of the total variation (Table 4, a). Separating *C. maritima* var. *maglicensis* and *C. serbica* into two groups decreased the variation among groups to 76.57% (Table 4, b). Collecting populations of *C. maritima* from Croatia and from Montenegro into one group decreased this value even more to 66.11% of the total variation (Table 4, c). Upon the

Table 3 Distribution of AFLP fragments and genetic diversity measures in populations of the *C. maritima* group, *C. glauca*, and *C. graeca*

Taxon	Pc	*N*	H_{Nei}	H_{Sh}	EF	DW	FT	FA
C. monteluccii	Tuscany1	2	*0.0043*	*0.0057*	0–0	6.43–6.95	41.47	41.00
	Marche 1	3	*0.0038*	*0.0046*	0–0	5.57–5.95	38.15	37.66
	Marche 2	3	*0.0057*	*0.0068*	0–0	5.71–6.38	40.66	39.98
	Tuscany2	2	*0*	*0*	0–0	6.85–7.23	43	43.00
C. fialae	Grude	5	0.0158	0.0181	1–2	5.93–7.25	41.06	39.20
	Klobuk	4	0.0161	0.0184	1–3	6.98–8.73	43.35	41.49
	Ružići	4	0.0225	0.0250	0–5	4.78–10.18	42.13	39.50
C. serbica + *C.* maglicensis*	Maglić 1	5	*0.0027*	*0.0030*	0–0	4.36–4.80	29.82	29.22
	Maglić 2	5	*0.0055*	*0.0061*	2–2	6.20–6.37	29.68	29.40
	Maglić 3	2	*0*	*0*	0–0	4.19–4.30	27	27.00
	Tara	3	*0*	*0*	2–2	6.29–6.39	30	30.00
C. rupestris	Čevo1	2	*0.0064*	*0.0085*	0–1	6.03–6.59	29.30	28.51
	Čevo2	4	0.0204	0.0233	0–2	5.97–8.25	30.35	28.01
	Grahovo	5	0.0192	0.0213	0–2	5.78–7.80	30.87	28.60
C. maritima—Montenegro	Risan	4	0.0102	0.0117	1–3	6.72–8.69	39.89	38.77
	Lovćen 1	4	0.0225	0.0257	1–4	5.37–9.70	39.15	36.47
	Ledenićе	4	0.0247	0.0282	0–3	5.62–7.80	37.92	34.98
	Lovćen 2	3	0.0114	0.0137	0–1	4.06–5.25	35.05	33.67
C. maritima—Croatia	Biokovo1	4	0.0381	0.0435	2–4	5.60–8.84	40.77	36.23
	Velebit 2	3	0.0172	0.0206	0–1	6.66–7.87	46.94	44.99
	Velebit 1	5	0.0330	0.0367	0–2	6.14–8.90	45.37	41.61
	Velebit 3	2	0.0150	0.0200	0–2	6.37–9.33	43.25	41.46
	Mosor 1	4	0.0188	0.0215	1–3	5.57–7.74	41.56	39.29
	Velebit 4	4	0.0236	0.0270	0–1	6.29–8.25	47.27	44.53
C. glauca	Lovćen	3	0.0114	0.0164	2–3	7.38–8.87	28.35	27.02
	Risan	3	0.0114	0.0164	0–1	4.61–6.09	26.96	25.64
	Maglić	3	0.0191	0.0273	2–6	6.59–11.28	28.27	25.98
	Spila	3	0.0267	0.0382	0–2	5.56–7.81	29.24	26.00
	Ledenićе	0					26	
	Cetinje	2	0.0107	0.0149	2–4	7.54–9.27	28.71	27.51
C. graeca	Grude	5	*0.0014*	*0.0016*	3–4	11.58–12.13	25.37	25.19
	Marche	4	*0.0021*	*0.0024*	1–2	9.71–10.25	23.75	23.49

Repeated resamplings were made to achieve the same sample size in each population (for details, see "Material and methods")

Pc population code (see Table 1); *N* number of investigated individuals per population; H_{Nei} Nei's gene diversity per population (high values are in bold, intermediate values in normal type, and low values in italics); H_{Sh} Shannon's diversity index (high values are in bold, intermediate values in normal type, and low values in italics); *EF (exclusive fragments)* fragments present in a given population, but absent in other populations; *DW* frequency-downweighted marker values; *FT (fragments total)* average total number of fragments per population; *FA (fragments average)* average number of fragments per individual

division of populations by geographical regions into two groups, Apennine and Balkan ones, genetic differentiation among the groups was only 22.70% of the total variation, while variation among populations in such cases accounted for 68.69% of the total (Table 4, d).

In analysing populations of *C. maritima* only, 57.71% of total genetic variation was accounted for among groups when populations of *C. maritima* from Croatia were separated from those from Montenegro (Table 4, e).

Analysis of the genetic diversity of populations and individual taxa (Table 3) showed that in three populations all individuals shared the same AFLP phenotype (*C. monteluccii*—Tuscany 2, *C.* maglicensis*—Maglić 3, and *C. serbica*—Tara). The lowest genetic diversity (low H_{Nei} and H_{Sh}) appeared in populations of *C. monteluccii* and *C. serbica* + *C.* maglicensis*. On the other hand, the highest genetic diversity was found in *C. fialae* and *C. maritima* (populations from both Croatia and Montenegro).

Table 4 Analysis of molecular variance (AMOVA) of AFLP data of the *Cardamine maritima* group, *C. glauca*, and *C. graeca*

Grouping	Source of variation	*df*	Sum of squares	Variance components	% of total variance
a					
[*C. graeca*] [*C. fialae*]	Among groups	7	1,888.921	18.93	77.04
[*C. monteluccii*]	Among populations	24	312.360	3.28	13.37
[*C. rupestris*]	Within populations	78	183.783	2.36	9.59
[*C. maritima* Croatia]					
[*C. maritima* Montenegro]					
[*C. serbica* + *C.* maglicensis*]					
[*C. glauca*]					
b					
[*C. graeca*] [*C. fialae*]	Among groups	8	1,895.804	18.70	76.57
[*C. monteluccii*]	Among populations	22	305.476	3.36	13.79
[*C. rupestris*]	Within populations	78	183.783	2.35	9.65
[*C. maritima* Croatia]					
[*C. maritima* Montenegro]					
[*C.* maglicensis*]					
[*C. serbica*]					
[*C. glauca*]					
c					
[*C. graeca*] [*C. fialae*]	Among groups	7	1,631.931	16.73	66.11
[*C. monteluccii*]	Among populations	24	569.349	6.22	24.58
[*C. rupestris*]	Within populations	78	183.783	2.36	9.31
[*C. maritima* Montenegro + Croatia]					
[*C. serbica* + *C.* maglicensis*]					
[*C. glauca*]					
d					
	Between groups	1	212.760	6.22	22.70
[Apennine Peninsula]	Among populations	29	1,988.521	18.81	68.69
[Balkan Peninsula]	Within populations	78	183.783	2.36	8.60
e					
C. maritima	Between groups	1	256.990	12.94	57.71
[Croatia]	Among populations	8	201.195	5.89	26.27
[Montenegro]	Within populations	27	96.950	3.59	16.02

Groupings are marked with square brackets

C. maglicensis* = *C. maritima* var. *maglicensis*

Discussion

Taxonomical implications

Originally, six taxa were described within the *C. maritima* group from the Balkan and Apennine Peninsulas, namely *C. monteluccii*, *C. maritima*, *C. fialae*, *C. maritima* var. *maglicensis*, *C. serbica*, and *C. maritima* "proles" *rupestris*. According to Flora Europea (Jones and Akeroyd 1993), Atlas Florae Europeae (Jalas and Suominen 1994), and most recent national floras from this area (Nikolić 1997; Trinajstić 1976; Josifović 1972; Pignatti 1982), only *C. maritima*, *C. monteluccii*, and *C. fialae* are recognised; the rest of the names are considered to be synonyms of *C. maritima*.

Molecular data presented in this study showed a clear pattern within the *Cardamine maritima* group. Italian populations of *C. monteluccii* are well separated from Balkan taxa. In a step forward from taxonomic confusion surrounding Balkan populations, our study confirms that five allopatric units—each with a clearly delimited and a rather restricted distribution range—can be easily recognised here. Molecular analyses showed that *C. maritima* var. *maglicensis* from the Maglić Mts. (Montenegro) and *C. serbica* from the Tara Mts. (Serbia) represent, genetically, the same taxon. The validly published name *C. serbica* (Pančić 1884) should be applied here; the name *C. maritima* var. *maglicensis* (Rohlena 1906) should be rendered into synonymy. While the names *C. serbica* (with

C. maritima var. *maglicensis* included in synonymy), *C. rupestris*, and *C. fialae* refer to clearly defined Balkan taxa, the name *C. maritima* applies to two genetically and geographically different entities. Because the populations from the type locality of *C. maritima* were not included in this study for technical reasons, the application of this name will require further attention, including the consideration of morphological aspects.

Our preliminary results (Kučera et al., unpublished data) show that the taxa supported by molecular markers are also differentiated morphologically. This is the subject of an ongoing study, which will also include populations from the type locality of *C. maritima*.

Relationships among taxa

There is low support for the internal nodes on the neighbour-joining trees, and it is hard to infer any relationships among taxa based on this information. In contrast to the situation for the internal nodes, individual taxa in all cases (except *C. serbica* and *C. maritima* var. *maglicensis*) gained high bootstrap support.

The NJ tree (Fig. 2), and in part the band-sharing data, indicate that populations of *C. fialae*, *C. monteluccii*, and two entities of *C. maritima* may be genetically closer to each other than to *C. rupestris* and *C. serbica*, which mostly occur in areas more distant from the coast. Moreover, *C. maritima* from Croatia shares the highest number of fragments with other taxa. There are two possible explanations for this pattern. The first scenario suggests that a formerly widespread taxon was isolated in refugial areas, where it evolved in situ into the taxa restricted to particular mountain ranges. This was followed by secondary contacts among taxa on both sides of the Adriatic Sea, involving all taxa but *C. rupestris* and *C. serbica.* During glacial periods, the level of the Mediterranean Sea was 100–200 m lower than that at present; many islands were interconnected or united to the mainland; and a wide landbridge filled the gap between the eastern and western Adriatic coasts (Colantoni et al. 1979, Voges 1995, Dawson 1992), thus providing opportunities for dispersal. Among the studied taxa, Croatian *C. maritima* is the most widespread, and it naturally has the greatest possibility of forming secondary contacts with other taxa. This is similar to the situation in rock partridges and *Campanula* (Randi et al. 2003; Park et al. 2006). Partridges in the Apennines and Albania-Greece are thought to have been connected by gene flow through a late Pleistocene Adriatic landbridge (Randi et al. 2003). Similarly, climatic fluctuations of the late Pliocene and Pleistocene have been invoked for trans-Adriatic exchange in *Campanula* (Park et al. 2006). The alternative scenario suggests that *C. rupestris* and *C. serbica* originated considerably earlier than taxa occurring on the Adriatic coast, resulting in the somewhat isolated position of these taxa. There is no visible support in our data for this alternative. Naturally, a combination of these scenarios is also possible. A more detailed study using chloroplast and nuclear DNA sequences, including single- or low-copy genes, may elucidate these relationships.

Although the PCoA diagram supports genetic homogeneity of the taxa (and two entities of *C. maritima*) recognised in this paper, their positions on the diagram are likely influenced not only by the actual relationships among taxa, but also by the number of fragments.

There is no evidence in our data (especially with respect to fragment sharing), which would support a hybrid or hybridogenous origin of *C. maritima*, *C. rupestris*, or *C. serbica*. Nevertheless, taking into account relationships depicted on the NJ tree that also included *C. glauca* and *C. graeca*, an ancient hybrid origin of *C. rupestris* and *C. serbica* involving *C. glauca* cannot be firmly excluded either (Fig. 3). Further studies using appropriate molecular markers may shed more light on this problem.

Influence of the glacial events

Detection of highly variable populations and populations containing rare fragments enable postulating hypotheses of long-term survival of these populations. The value of DW is expected to be high in populations isolated for a long period, in which rare markers should accumulate due to mutations, whereas newly established populations are expected to exhibit low values. The number of unique fragments also supports the long-term survival of populations in their present day locations.

According to Médail and Diadema (2008), the Velebit Mts., S. Bosnia/Biokovo, and Montenegro are among the fifty major refugia of Mediterranean plants. This region coincides with the distribution areas of both entities of *C. maritima*, *C. rupestris*, and partly also *C. fialae* and *C. serbica* and suggests that populations of those taxa may have survived glaciation events in their current localities and were subject to altitudinal rather than latitudinal vegetation movements.

Cardamine serbica populations have low values of both indices of genetic diversity, DW as well as unique fragments, when all the populations are considered together. Nevertheless, in two of four analysed populations, the number of unique fragments and DW values are slightly higher. As both these populations occur in mountain gorges, which represented the refugial biotopes (Thompson 2005), we can speculate that the populations are relic ones. This is particularly true for the upper part of the Piva river valley (population Maglić 2). We can also speculate that, during their evolutionary history, populations of *C. serbica* experienced a serious bottleneck, which may account for their generally low genetic diversity.

Populations of both entities of *Cardamine maritima*, occurring on the open mountain slopes and gorges oriented towards the sea, are genetically more variable. This is particularly true for the Croatian populations from Biokovo and Velebit. This variability is apparent when considering individual populations, as well as when calculating corresponding values for *C. maritima* from Croatia as a whole and for *C. maritima* from Montenegro as a whole. One can speculate that the higher values for *C. maritima* from Croatia are influenced by the number of populations analysed, but the values also reflect the considerably larger distribution area of this entity, compared to the other taxa studied here (except for *C. monteluccii*). Higher diversity in this case might be caused, not only by the relic status of these populations, but also by the possibility of making secondary contacts, especially during the glacial periods. Compared to *C. maritima*, populations of *C. serbica* were most likely isolated from other species of the complex for a much longer time.

Although the populations of *C. fialae* occur in hills that are some distance from the coast and in different ecological conditions (they grow shaded in the forest rather than on sunny slopes of the sea coast as *C. maritima*), *C. fialae* populations exhibit considerable genetic variability; it is likely that their distribution area was not considerably influenced by the glacial events. A peculiar feature of this taxon is a low number of exclusive fragments for the species (although this is not completely true on the population level).

Somewhat in-between with respect to genetic variation are populations of *Cardamine rupestris*. They occupy an area, which is distant from the coast and grow shaded in the forest in hilly ranges, but not to the same extent as *C. serbica*, and we can expect some secondary contacts of these populations with other taxa of the group.

Both diversity indices—the low obtained values of DW, as well as the number of unique fragments—fail to indicate the relict origin of the analysed populations of *C. monteluccii*. Postglacial re-colonisation from adjacent regions is a more plausible scenario, but more robust sampling (including the southern part of the distribution area) is desirable for elucidating the phylogeography of this taxon.

Similarly, both diversity index and DW of *Cardamine graeca* accessions (species widespread across the Mediterranean region) suggest a decrease in genetic variation within the analysed populations. On the other hand, the number of exclusive fragments was significantly higher. Long-term spatial separation and the at least partial autogamy found in *C. graeca* (Kučera, unpubl.) may be responsible for the presence of the detected exclusive fragments.

The large genetic variation of five populations of *Cardamine glauca* from Montenegro suggests the refugial character of the sampled area (gorge of the river Piva and mountains in Kotor Bay), though the still unclear taxonomy (diversification within the taxon and the presence of further hidden, not-yet-recognised entities, Lakušić, personal communication) of this polymorphic taxon may underlie the increased genetic richness of the populations.

From the evidence discussed above, we can conclude that the majority of Balkan populations exhibit features of genetic variability that enable us to hypothesise that they belong to relic populations. In spite of the possibility of secondary contacts among taxa (or groups of populations) during glaciation periods, it is very likely that the overall distribution areas of these taxa were not influenced by the glacial events. This is in sharp contrast to the phylogeographic history of many other European species of the genus *Cardamine*, particularly polyploid ones, which show patterns of genetic variation indicating that their evolutionary history and distribution areas were considerably influenced by the glacial events (e.g., Franzke and Hurka 2000).

Conservation measures

The results of our analysis show that apart from currently recognised taxa, namely *C. maritima* and *C. monteluccii*, there are several units, which are strongly genetically differentiated. Some of them, namely *C. fialae* and *C. serbica*, occupy only restricted distribution areas, and appropriate conservation measures for their protection should be taken. This is in accordance with the opinion of Moritz (1994), who stressed the need to recognise not only conservation units based on predominantly morphological criteria, but also those concerned with historical population structure and DNA phylogeny. Nevertheless, our ongoing studies are also likely to reveal morphological differences among at least some of the abovementioned units, and they can be treated as species in the traditional sense.

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