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Non-concordant phylogeographical patterns of three widely codistributed endemic Western Balkans lacertid lizards (Reptilia, Lacertidae) shaped by specific habitat requirements and different responses to Pleistocene climatic oscillations

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Abstract

The Balkan Peninsula is a hot spot for European herpetofaunal biodiversity and endemism. The rock climbing lizards *Dalmatolacerta oxycephala* and *Dinarolacerta mosorensis* and the ground-dwelling Dalmatian wall lizard *Podarcis melisellensis* are endemic to the Western Balkans, and their ranges largely overlap. Here, we present a comparative phylogeographical study of these three species in the area of their codistribution in order to determine the level of concordance in their evolutionary patterns. Phylogenetic analyses were performed based on two mitochondrial genes (*cytochrome b* and *16S rRNA*), and a molecular clock approach was used to date the most important events in their evolutionary histories. We also tested for correlations regarding genetic differentiation among populations and their geographical distances. For all three species, a significant correlation between genetic and geographical distances was found. Within *D. oxycephala*, two deeply separated clades ('island' and 'mainland clade'), with further subdivision of the 'mainland clade' into two subclades ('south-eastern' and 'north-western'), were found. High sequence divergences were observed between these groups. From our data, the time of separation of the two main clades of *D. oxycephala* can be estimated at about 5 mya and at about 0.8 mya for the two subclades of the mainland clade. Within *D. mosorensis*, coalescence time may be dated at about 1 mya, while *D. mosorensis* and *D. montenegrina* separated around 5 mya. The results imply the existence of complex palaeo-biogeographical and geological factors that probably influenced the observed phylogeographical patterns in these lacertid species, and point to the presence of numerous glacial/interglacial refugia. Furthermore, the observed cryptic genetic diversity within the presently monotypic species *D. oxycephala* prompts for a revision of its taxonomic and conservation status.

Key words: *Podarcis melisellensis* – *Dalmatolacerta oxycephala* – *Dinarolacerta mosorensis* – mitochondrial DNA – cryptic diversity – microrefugia

Introduction

The majority of present-day European species are supposed to have survived the extreme Pleistocene climatic oscillation in one of the three main European southern refugia: Iberian, Apennine and Balkan peninsulas (de Lattin 1967; Taberlet et al. 1998; Hewitt 1999). The multiple events of range retractions and subsequent expansions have often caused long-term isolation, bottlenecks and extinction of marginal populations. These processes have left complex genetic imprints on the populations of the species, thereby shaping their present phylogeographical structure (Hewitt 1996, 2000, 2004; Avise 2000). The main goal of comparative phylogeography is to search for patterns of phylogeographical concordance among lineages of multiple co-occurring species, which would indicate the influence of common historical factors (Taberlet et al. 1998). Recent phylogeographical studies of different animal taxa (e.g. Kryštufek et al. 2007; Previšić et al. 2009; Ferchaud et al. 2012) revealed that the 'refugia within refugia' concept (Gómez and Lunt 2007), originally described for the Iberian Peninsula, is also valid for the Balkans. However, although the Balkan region is richer in species and palaeoendemics in comparison with the Iberian and Apennine peninsulas, the number of phylogeographical studies (particularly comparative ones) of Balkanic fauna is still scarce (Hewitt 2011). The Balkan Peninsula is a hot spot for European reptile biodiversity and herpetofaunal endemism, harbouring 70 reptile species, 15 of them being endemic (Džukić and Kalezić 2004). Even after the Messinian salinity crisis (which ended 5.33 mya), the geological history of the Balkan was complex. The uplifting of the Dinarides caused pronounced changes in the relief, and alterations of ingressive and regressive sea phases during the Pliocene and Pleistocene were changing the shape of the coastline (De Giuli et al. 1987; Marović et al. 1999; Kuhlemann 2003, 2007). Nevertheless, it is generally thought that for most of this time period, all east Adriatic Islands and the mainland were part of the same landmass until they finally became separated in the course of the Holocene eustatic sea-level rise. The complex topography of the region provided the existence of numerous environmental niches that preserved biodiversity during Pleistocene climate extremes (Griffiths et al. 2004). Genetically strongly differentiated palaeoendemic species that have persisted for a long time within small geographical regions provide a useful model for inferring the presence of microrefugia and differentiation centres within larger refugial areas (Kryštufek et al. 2007).

Three species of lacertid lizards were investigated in this study: the sharp-snouted rock lizard *Dalmatolacerta oxycephala* (Duméril & Bibron, 1839), the Mosor rock lizard *Dinarolacerta mosorensis* (Kolombatović, 1886) and the predominately ground-dwelling Dalmatian wall lizard *Podarcis melisellensis* (Braun, 1877). They are endemic in the Western Balkans, and their ranges largely overlap (Fig. 1a, b and c; source Crnobrnja-Isailović et al. 2009a,b; Ajtić et al. 2009). *Dalmatolacerta oxycephala* has a relatively small but pretty continuous distribution range (Crnobrnja-Isailović et al. 2009b) (Fig. 1a). It extends along the east Adriatic coast (including most off-shore islands south of Split as well as the more remote islands of the Vis and Lastovo archipelagos) from Šibenik (Croatia) south to the Albanian border. In addition to Croatia and Montenegro, this species also inhabits large parts of Herzegovina (Schreiber 1891; Bischoff 1984; Arnold 1987). Its altitudinal range extends from sea level to about 1600 m a.s.l. (Arnold 1987). In the coastal region and

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islands, *D. oxycephala* is broadly sympatric with *P. melisellensis,* which inhabits the coastal mainland and most of the islands of the eastern Adriatic region (Fig. 1c), ranging from the area of Monfalcone (Italy) in the north-west to Tuzi and Shkodër (Albania) in the south-east (Tiedemann and Henle 1986). The range of *P. melisellensis* extends from sea level up to 1400 m a.s.l. (Ajtić et al. 2009). In contrast to the rather continuous distributions of the two former species, *D. mosorensis* has a severely fragmented distribution range in the south-western Dinaric mountain karst of southern coastal Croatia, southern Bosnia-Herzegovina and Montenegro (Fig. 1b) and is restricted to altitudes above 450–550 m a.s.l. (Arnold 1987; Crnobrnja-Isailović and Džukić 1997). Although frequently sympatric, the three species differ in their ecological requirements and habitat preferences. While *D. oxycephala* and *D. mosorensis* occur largely on rocky surfaces taking refuge in the crevices of large rocks or rock piles, *P. melisellensis* inhabits diverse herbaceous or bushy habitats (from open dry woodland to open slopes with patches of long grass) where it takes refuge in holes and vegetation (Arnold 1987). In comparison with *D. oxycephala,* which usually occurs on sun-exposed surfaces, *D. mosorensis* prefers cooler, more humid and shady habitats. It also does not climb as high above the ground as *D. oxycephala* (Arnold 1987). In his study on the altitudinal distribution of lizards on Biokovo Mt., Schmidtler (1999) reported that *D. oxycephala* as well as *D. mosorensis* inhabit the same rocky places but they are separated altitudinally, *D. oxycephala* being distributed up to 1050 m and *D. mosorensis* above 1200 m a.s.l.

Several previous studies showed that the phylogeographical pattern of the Balkan lacertid species was shaped by the influence of various historical factors such as geological events (i.e. emergence of geographical barriers), climatic oscillations, over-water dispersal and unintentional anthropogenic colonization (Podnar et al. 2004, 2005; Poulakakis et al. 2005; Böhme et al. 2007; Ljubisavljević et al. 2007). Specifically, four distinct phylogeographical regions were revealed for *P. melisellensis*: Lastovo archipelago (1), Vis archipelago (2), mainland with offshore islands north (3) and south (4) from the Neretva River (Fig. 1d, Podnar et al. 2004).

The taxonomy of rock lizards recently underwent substantial modifications. *D. mosorensis* and *D. oxycephala* were classified in the past as species of *Archaeolacerta* (Mertens, 1921), which was considered as a subgenus of *Lacerta* or as a distinct genus. Recently, Arnold et al. (2007) put them in two separate newly described monospecific genera, *Dinarolacerata* and *Dalmatolacerta*, respectively. Both species were originally regarded as monotypic, although pronounced morphological and genetic differences between local populations have been reported for *D. mosorensis* (Carranza et al. 2004; Arnold et al. 2007). Recently, based on these features, Ljubisavljević et al. (2007) described a second species of the genus *Dinarolacerata, D. montenegrina*, from the Prokletije mountain massif in Montenegro.

Dalmatolacerta oxycephala is still the only species within its genus, although two remarkably different phenotypes can be distinguished: a more or less light grey-coloured form and a much darker, sometimes entirely black form (‘*tomasinii*’) (Bischoff 1984). The dark ‘*tomasinii*’ form occurs primarily in the south-eastern parts of the species’ area, especially in the mountainous areas of east Herzegovina and Montenegro (Schreiber 1891). These two forms seem to differ not only in coloration but also in some additional morphological (pholidotic) characters. According to Bischoff (1984), a split of the species into two subspecies would be morphologically and historically justified.

In contrast to the above-mentioned monotypic species, *P. melisellensis* was considered to be a polytypic species with 20 described subspecies (Tiedemann and Henle 1986). However, a comprehensive phylogeographical study based on three mitochondrial DNA markers (Podnar et al. 2004) revealed only three deeply separated clades within the species: the ‘fiumana’ lineage comprising mainland and nearby islands, the ‘Lastovo’ lineage restricted to the Lastovo archipelago and the ‘melisellensis’ lineage encompassing the populations of three adjacent island groups in southern Dalmatia: the Island Vis with nearby islets, the Island Biševo and the Svetac Island group. Therefore, only three subspecies were proposed. In addition, within the ‘fiumana’ lineage, two clearly separated sublineages were found encompassing the populations north and south of the ancient course of the River Neretva (‘northern fiumana’ and ‘southern fiumana’, respectively).

The codistribution of the three lacertid species described above (*P. melisellensis*, *D. oxycephala* and *D. mosorensis*) offers an interesting possibility for a comparative phylogeographical study regarding the impact of common historical factors and biogeographical boundaries on their evolutionary histories with respect to their specific habitat requirements and ecology.

The aim of this investigation was to answer the following questions: (1) Is there any concordance in phylogeographical patterns of *P. melisellensis, D. oxycephala* and *D. mosorensis,* which would indicate a similar response to climatic changes during quaternary glaciations and eventually enable us to infer possible common glacial refugia? (2) Is there cryptic genetic diversity within *D. oxycephala*? (3) Do the two colour morphs represent genetically divergent lineages? Such finding would support Bischoff’s (1984) suggestion to recognize two subspecies within *D. oxycephala.* In order to answer these questions, we performed a phylogeographical analyses of these species. We sequenced parts of the mitochondrial *cytochrome b* (*cyt b*) and *16S rRNA* genes and combined the new data with those published earlier for *P. melisellensis* (Podnar et al. 2004). Furthermore, using a molecular clock analysis, we attempted to date the most important events in their evolutionary histories. We also discuss the implication of our findings for taxonomy and conservation.

Materials and Methods

DNA amplification and sequencing

A total of 27 samples of *Dalmatolacerta oxycephala* from 21 localities and 10 samples of *Dinarolacerta* from seven localities were analysed (Table 1, Fig. 1d). Although not particularly dense, the sampling largely covered the ranges of species. The samples of *D. oxycephala* were chosen in such a way that each area inhabited by one of the main *P. melisellensis* lineages was represented (Fig. 1d). The samples of *D. oxycephala* from two localities (Mt. Lovćen and Korita) belonged to the dark form (Table 1). Sequence data of *P. melisellensis* from our previous study (comprising a total of 73 individuals, Podnar et al. 2004) were combined with three new samples of *P. melisellensis* (Table 1). Altogether, the sequences from 113 individuals were analysed in the study. Total genomic DNA was extracted by a standard phenol-chloroform protocol (Sambrook et al. 1989) from deep-frozen or ethanol-preserved soft tissues or tail tips.

Fragments of two mitochondrial genes, *cyt b* (964 bp) and *16S rRNA* (about 500 bp), were amplified via polymerase chain reaction (PCR). For the *16S rRNA* fragment amplification and reamplification, the following primers were used: L-1625 (5′ GTGGGCCTAAAAGCAGCCAC 3′, Reeder 1995) and H-2456 (5′ CCGGTCTGAACTCAGATCACG 3′, Heise et al. 1995). PCR conditions were the same as described in Podnar et al. (2004). Sequencing primer was L-1926 (5′ CGCCTGTTTACCAAAAACAT 3′, Knight and Mindell 1993). For *cyt b*, different primer pairs were used for *D. oxycephala*, *Dinarolacerta* and *P. melisellensis*, respectively: in *D. oxycephala*, *cyt b* was amplified with the primers: L-GluLK (5′ AA CCGCTGTTGTCTTCAACTA 3′) and H-ProLK (5′ AGTTTTGGGGG CTAGTGATGG 3′), and, if necessary, reamplified with H15149 (5′ CCATCCAACATCTCAGCATGATGAAA 3′; Kocher et al. 1989; modi-

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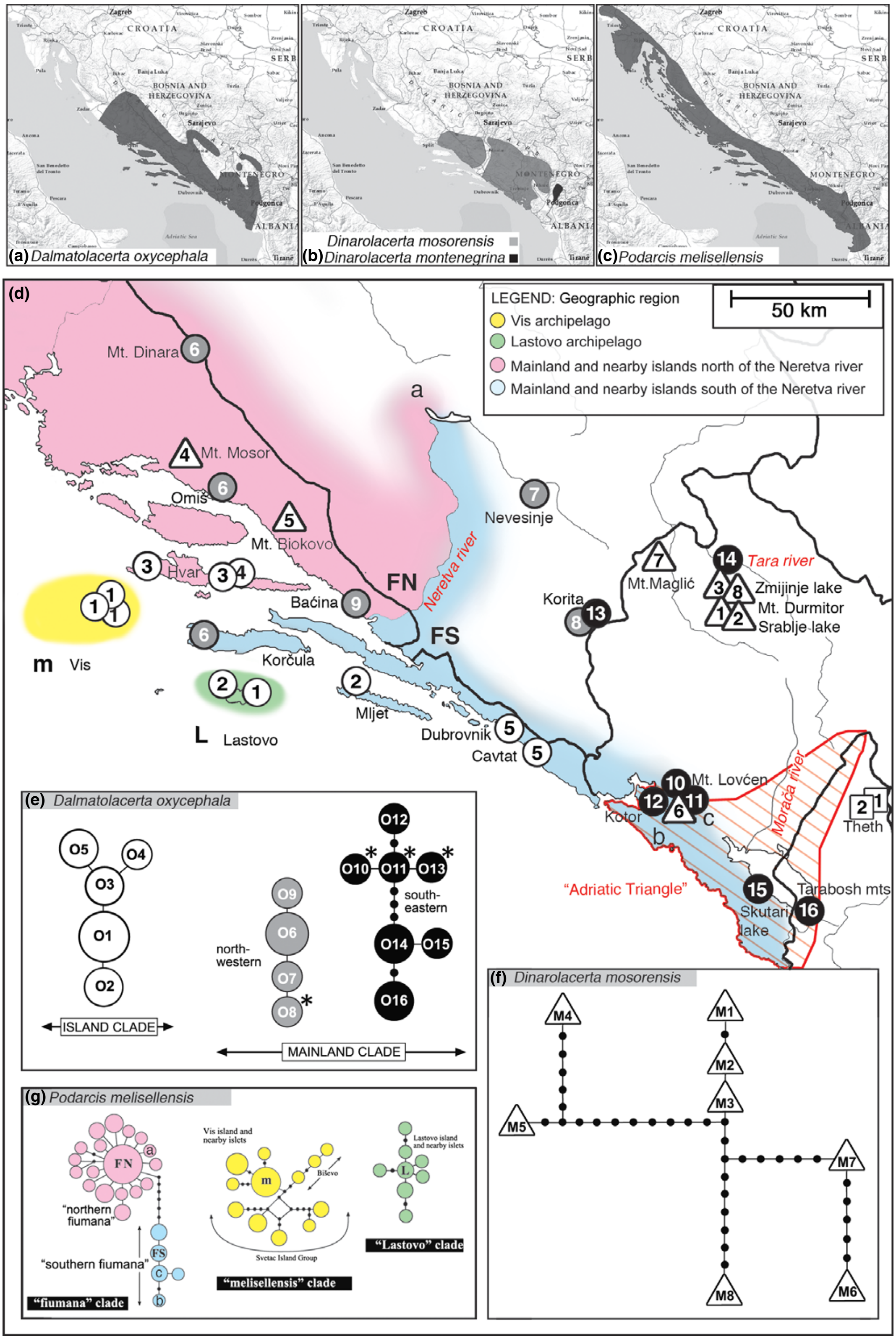

Fig. 1. Distribution ranges of (a) *Dalmatolacerta oxycephala*, (b) *Dinarolacerta mosorensis* and *Dinarolacerta montenegrina* and (c) *Podarcis melisellensis*. (d) Sample localities and haplotypes (see Table 1) of *D. oxycephala*, *D. mosorensis* and *D. montenegrina* are indicated with circles, triangles and squares, respectively. Three localities of *P. melisellensis* are indicated with letters a, b and c. Four geographical areas inhabited by distinct *P. melisellensis* lineages are indicated with different colours (based on Podnar et al. 2004). Acronyms m, L, FN and FS correspond to 'melisellensis', 'Lastovo', 'northern' and 'southern fiumana' lineages, respectively. (e–g) TCS parsimony networks based on *cyt b* sequences of *D. oxycephala*, *D. mosorensis* and *P. melisellensis* (modified after Podnar et al. 2004). Small black circles represent missing intermediates. In e and g, the size of ovals corresponds to haplotypes frequencies. Haplotypes found in the dark ('*tomasinii*') form of *D. oxycephala* are indicated with asterisks (*). In (d) and (e), white, grey and black circles correspond to 'island clade' and 'north-western' and 'south-eastern' subclades of the 'mainland clade' of *D. oxycephala*, respectively. 'Adriatic Triangle' is indicated in d (after Džukić and Kalezić 2004).

Table 1. Individuals analysed in the present study, geographical origins, collection numbers (Tissue collection of the Natural History Museum Vienna; additional NHMW numbers refer to the Herpetological collection of the Natural History Museum Vienna), combined *cyt b* + *16S rRNA* mitochondrial haplotypes and GenBank accession numbers are given for samples of *Dalmatolacerta oxycephala* and *Dinarolacerta*. Individuals of the dark form of *D. oxycephala* (‘*tomasinii*’) are indicated (*). For the three newly sequenced *P. melisellensis* samples, only *cyt b* haplotypes were sequenced (**). Letters a, b and c correspond to their sampling localities (Fig. 1d). The remaining *cyt b* and *16S rRNA* haplotypes used in this study are listed in Podnar et al. (2004) (AY185009-AY185091, AY185097).

Species	Locality	Collection number	Combined haplotype	GenBank Acc. No.	
				16S rRNA	*cyt b*
Dalmatolacerta oxycephala	HR: Vis Island, Rukavac	OW2 (NHMW 36327 : 2)	O1	AY278192	AY278199
		OW3 (NHMW 36327 : 3)	O1	KF373653	KF373613
	HR: Greben islet (Vis)	OW5	O1	KF373654	KF373614
	HR: Ravnik islet (Vis)	OW7	O1	KF373655	KF373615
	HR: Lastovo Island, Žabarje	OW8 (NHMM 36327 : 7)	O1	AY278189	AY278196
	HR: Mljet Island, Polača	OW11	O2	KF373658	KF373618
	HR: Mrčara Island	OW12	O2	KF373659	KF373619
	HR: Hvar Island, Hvar city	OR3	O3	KF373662	KF373622
	HR: Hvar Island	OR1 (NHMW 35849 : 1)	O3	KF373660	KF373620
		OR2 (NHMW 35849 : 2)	O4	AF440616	AY278195
	HR: Cavtat	NV3 (NHMW 36338 : 1)	O5	AY278187	AY278193
	HR: Dubrovnik	NV4 (NHMW 36338 : 2)	O5	AY278188	AY278194
	HR: Korčula Island, Vela Luka	OW10	O6	KF373657	KF373617
	HR: Mt. Dinara	OX15	O6	KF373671	KF373631
	HR: Cetina valley near Omiš	OX27	O6	KF373678	KF373638
	BIH: Nevesinje	OX14	O7	KF373670	KF373630
	BIH: Korita	OX19*	O8	KF373675	KF373635
		OX18*	O13	KF373674	KF373634
	HR: Baćina near Ploče	OX16	O9	KF373672	KF373632
	MNE: Mt. Lovćen	OX7* (NHMW 37261 : 5)	O10	AY278190	AY278197
		OX8* (NHMW 37261 : 6)	O11	AY278191	AY278198
	MNE: Kotor	OX17	O12	KF373673	KF373633
	MNE: Tara River canyon	OX11	O14	KF373667	KF373627
		OX12	O14	KF373668	KF373628
	MNE: Skutari Lake, Donji Murići	OX13	O15	KF373669	KF373629
	AL: Tarabosh mts	OX23	O16	KF373676	KF373636
		OX25	O16	KF373677	KF373637
Dinarolacerta mosorensis	MNE: Mt. Durmitor, Srablje Lake	MO5	M1	KF373679	GQ142130
		MO6	M2	KF373680	KF373640
	MNE: Mt. Durmitor, Zmijinje Lake	MO9	M3	KF373681	KF373641
		MO18	M8	KF373686	KF373646
	HR: Mt. Mosor	MO12	M4	KF373682	KF373642
	HR: Mt. Biokovo	MO15	M5	KF373685	KF373645
	MNE: Mt. Lovćen	MO21	M6	KF373687	KF373647
	MNE: Mt. Maglić	MO22	M7	KF373688	KF373648
Dinarolacerta montenegrina	AL: Theth	MO13	G1	GQ142101	GQ142141
		MO14	G2	KF373684	KF373644
Podarcis melisellensis	BIH: Ramsko Lake	NHJS1	FN18**(a)	–	KF373649
	MNE: Mt. Lovćen	NCGL1	FS1** (b)	–	KF373650
	MNE: Budva	NCGC1	FS5** (c)	–	KF373651

fied) and H-ProLK. PCR conditions were as described in Podnar et al. (2004). Sequencing was performed with the primers OX14700L (5′ AGGTACCTCTATAATTCACC 3′) and OX14850H (5′ GGGTCTCCTA GTAGGTTAGG 3′). In *D. mosorensis* and the three new samples of *P. melisellensis*, the complete *cyt b* gene was amplified using the primers L-14132 (5′ ATTCAACTATTAAAACCTCTAATG 3′) and H-15425 (5′ GGTTTACAAGACCAGTGCTTT 3′) (Podnar et al. 2004). PCR products were extracted from the agarose gel (QIAquick Gel Extraction Kit, QUIAGEN, Hilden, Germany), cloned using the TOPO TA Cloning Kit (Life Technologies, Carlsbad, CA, USA) and sequenced using universal M13 primers.

Sequencing was carried out by MWG-BIOTECH (Ebersberg, Germany) or AGOWA (Berlin, Germany). GenBank accession numbers are given in Table 1.

Phylogenetic analyses

Sequences obtained in this study were deposited in GenBank under the accession numbers listed in Table 1. Previously published sequences of *P. melisellensis* (Podnar et al. 2004) can be found under GenBank numbers AY185009-AY185091 and AY185097. Among these, the following sequences were selected as representatives for the geographical clades in Fig. 2: *P. melisellensis fiumanus*, ‘north fiumana’ (NHJS; AY185010), *P. m. fiumanus*, ‘southern fiumana’ (AY185049, AY185012), *P. melisellensis* ‘Lastovo’ (AY185036, AY185015) and *P. m. melisellensis* (AY1850971, AY185018). Published *cyt b* and *16S rRNA* sequences of *Hellenolacerta graeca* (GQ142128, GQ142100) were used to root the tree. Furthermore, sequences of species closely related to *Dinarolacerta* (*Algyroides nigropunctatus*, GQ142132, AY942795) and to *P. melisellensis* (*Podarcis siculus*: *P. s. siculus* (NC_011609), *P. s. campestris* (AY185094, AY184996) were retrieved from GenBank and included in the analyses. Outgroups were selected according to the Lacertinae phylogeny published by Pavličev and Mayer (2009).

Phylogenetic analyses were conducted using three different methods of phylogenetic inference: maximum parsimony (MP) and maximum likelihood (ML) as implemented in PAUP (v 4.0b10, Swofford 2002), and Bayesian inference (BI) as implemented in MrBayes (version 3.1.2, Ronquist and Huelsenbeck 2003). A partition-homogeneity test (Farris et al. 1995) with 100 replicates as implemented in PAUP (version 4.0b10, Swofford 2002) was performed to test for potential heterogeneity in the phylogenetic signal among *cyt b* and *16S rRNA* data sets. Since no significant incongruity between the two gene fragments was revealed

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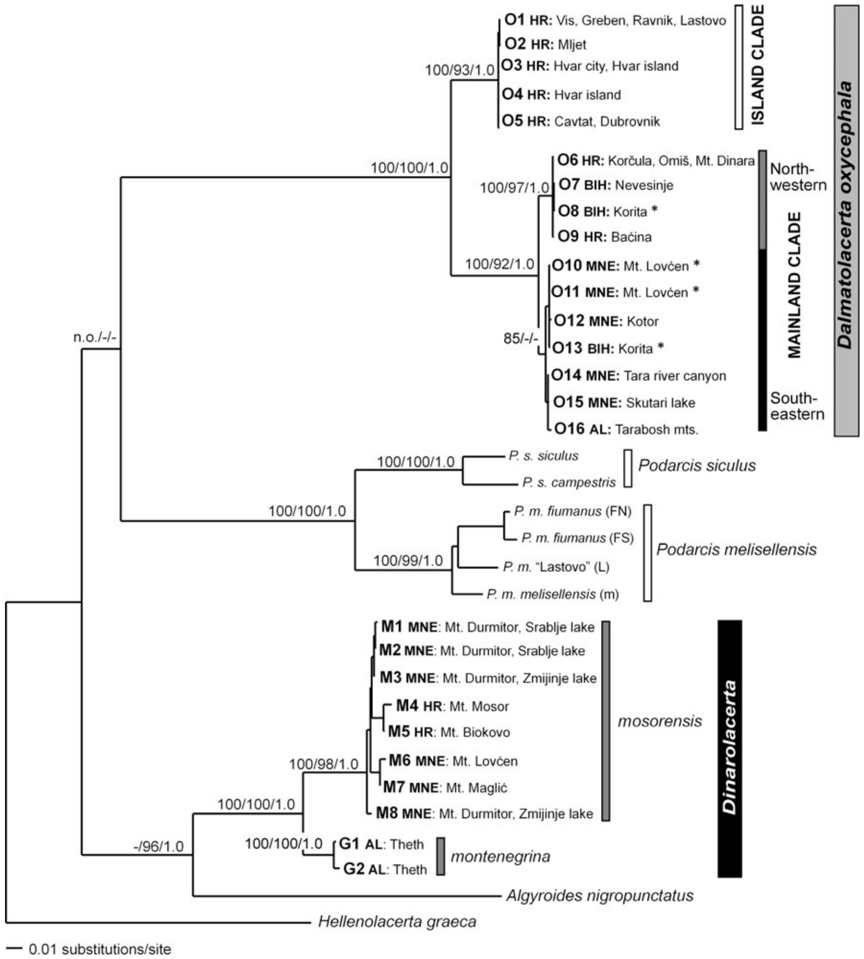

Fig. 2. Phylogram derived from ML analysis of concatenated *cyt b* and *16S rRNA* sequences. Numbers at the nodes represent support values: MP/ML/Bayesian. Bootstrap values <70 and Bayesian posterior probabilities <0.9 are not indicated. n.o. = node not obtained. Haplotypes found in the dark (‘*tomasinii*’) form of *D. oxycephala* are indicated with asterisks (*).

(p = 0.25), all phylogenetic analyses were performed on the concatenated sequences (length of the alignment: 1476 bp).

The optimal model of sequence evolution (TPM2uf+G) was selected under the Akaike’s information criterion (AIC) using jModelTest 0.1.1 software (Guindon and Gascuel 2003; Posada 2008) and implemented in ML and BI analyses. For MP analysis, alignment gaps were treated as missing data. MP and ML analyses were performed with a heuristic search with 100 and 10 random addition replicates, respectively, and tree bisection–reconnection (TBR) branch swapping algorithm. Nodal support was assessed with 1000 and 100 bootstrap (BS) replicates for MP and ML trees, respectively. BI trees were calculated from 3 million generations by sampling trees every 100th generation and discarding the first 20% of the trees as a burn-in.

Finally, since the phylogenetic networks are often better suited than tree-building algorithms to display the relationships at the intraspecific level (Posada and Crandall 2001), we constructed statistical parsimony networks based on *cyt b* sequences under a 95% parsimony connection limit using TCS v. 1.21 (Clement et al. 2000) for each of the following species: *D. oxycephala*, *D. montenegrina*, *D. mosorensis* and *P. melisellensis*.

Genetic distances

Intraspecific (*Dalmatolacerta oxycephala*, *Podarcis melisellensis*, genus *Dinarolacerta*) as well as interspecific (genus *Dinarolacerta*) uncorrected pairwise genetic distances (p-distances) were calculated for each gene fragment separately using Mega v.5 (Tamura et al. 2011). To test for significant relationships between genetic differentiation among populations and their geographical distances (‘isolation by distance’ or IBD, Rousset 1997), a Mantel’s test (Mantel 1967) was performed with 30.000 permutations using the IBDWS 3.15 software (Jensen et al. 2005) based on a genetic distance matrix (p-distances) of *cyt b* sequences. A geographical distance matrix was created based on geographical coordinates by using Geographic Distance Matrix Generator (Ersts 2011).

Molecular dating

The major cladogenetic events of *Dalmatolacerta* and *Dinarolacerta* were dated separately using the *cyt b* data and the program Beast v1.7.4 (Drummond et al. 2012). The molecular clock hypothesis was tested using a maximum-likelihood approach implemented in Mega v.5 (Tamura et al. 2011). Since the molecular clock hypothesis has been accepted for *Dalmatolacerta*, but rejected for *Dinarolacerta*, we applied Bayesian strict and relaxed uncorrelated clock methods for these data sets, respectively. In the absence of fossil data of lacertid lizards, calibration of evolutionary rates is highly speculative. Therefore, we used two published divergence rates for *cyt b* (Paulo et al. 2001, 2008): 1.7% and 2.5% pairwise sequence divergence/million years. However, since this widely used *cyt b* molecular clock for Lacertidae was calibrated on the basis of a ‘short’ fragment (307 bp, positions 99–405 in the *cyt b* gene) (Thorpe et al. 1994; González et al. 1996), p-distances were also calculated for this fragment in order to compare the evolutionary rate of this part of the gene with the part used in the analyses in this study (positions 180–1143, ‘long’ fragment). The evolutionary rate of the ‘long fragment’ appeared to be 1.2 times faster compared with the ‘short’ one; therefore, we applied this correction to mean distances (resulting in 2.04% and 3.0% pairwise sequence divergence/million years) in subsequent molecular dating analysis. Therefore, we applied the average substitution rate of 0.013 mutations/site/million years with a standard deviation of 0.002 to estimate divergence times.

For both data sets, the HKY+G substitution model and a coalescent constant size tree prior were used. The analyses were run for 10 million generations, sampling every 1000 generations, and the final tree was produced with TreeAnnotator v.1.7.4 (Drummond et al. 2012) from the 9000 trees sampled after the burn-in. The convergence of runs, ESS and burn-in values were checked by Tracer v1.5 (Rambaut and Drummond 2009).

Results

Phylogenetic analyses

An overview of the relationships within the taxa analysed is illustrated in the ML tree in Fig. 2, which also includes support values of MP and BI analyses. All algorithms resulted in a mostly congruent overall topology. While the ML and BI analyses yielded the same tree topology, the only major difference between them and the MP analysis concerned the position of the *Podarcis* clade. In the ML and Bayesian trees, it clustered with *Dalmatolacerta oxycephala*, while in the MP tree, it appeared as a sister group of the *A. nigropunctatus* + *Dinarolacerta* clade. As the tree does not include several of the related genera, it is not intended to represent a comprehensive phylogeny of the group. However, it should allow a comparison of the relationships and genetic distances within and between species and clades of the taxa analysed.

Within *D. oxycephala*, two deeply separated clades were found. One, the ‘island clade’, is distributed mostly on Adriatic islands, but also in the cities of Dubrovnik and Cavtat in southern Dalmatia. The other one, the ‘mainland clade’, occurs over most of the mainland range of the species and, additionally, on the island of Korčula. The ‘mainland clade’ is divided into two subclades, a north-western one in Croatia and Herzegovina and a south-eastern one in Montenegro, extending into the north-westernmost part of Albania. Those two subclades are in contact in easternmost Herzegovina near the montenegrin border (Korita, Fig. 1d).

In the parsimony network, all three *D. oxycephala* (sub)clades appear as clearly separated, unconnected groups (Fig. 1e). A further subdivision can be seen in the south-eastern subclade in which eastern and western populations are separated by four mutational steps. In contrast, all *D. mosorensis* samples cluster in a single clade; nevertheless, most of the haplotypes are separated by high numbers of mutational steps (Fig. 1f). TCS analysis of *P. melisellensis* resulted in the same three subnetworks as already described in Podnar et al. (2004) and all new samples clustered within the ‘fiumana’ lineage (Fig. 1g).

The intra- and interspecific p-distances are given in Table 2. The sequence divergences of *cyt b* within all three (sub)clades of *D. oxycephala* are very small, while those between the main clades of *D. oxycephala* (9.4–10.1%) are even higher but on the same order of magnitude as the difference between the two species of *Dinarolacerta* (6.9–8.4%). For comparison, the sequence divergences between the main clades of *P. melisellensis* (‘fiumana’, ‘melisellensis’ and ‘Lastovo’) ranged from 4.5 to 6.5% (Table 2).

Isolation by distance

A positive and significant correlation (99% confidence level) between genetic and geographical distances was revealed by IBDWS analysis for all three species (*P. melisellensis*: $r = 0.169$, $p = 0.0001$; *D. oxycephala*: $r = 0.352$, $p = 0.0006$; *D. mosorensis*: $r = 0.77$, $p = 0.0016$). However, the shapes of Mantel test plots for *P. melisellensis* and *D. oxycephala* clearly indicate the presence of barriers to gene flow, while the *D. mosorensis* plot indicates continuous populations under isolation by distance (Fig. S1).

Table 2. Observed ranges of uncorrected pairwise sequence divergences (in percentages): (A) within and between (in bold) main clades of *Dalmatolacerta oxycephala,* (B) within and between (in bold) subclades of the ‘mainland clade’ of *Dalmatolacerta oxycephala,* (C) within the genus *Dinarolacerta,* (D) within and between (in bold) main clades of *Podarcis melisellensis.* *After Podnar et al. (2004).

	cyt b		*16S rRNA*	
Dalmatolacerta oxycephala				
(A)	island clade	mainland clade	island clade	mainland clade
island clade	0.1–0.3		0.0	
mainland clade	**9.4–10.1**	0.1–2.2	**2.0–2.6**	0.2–0.8
(B)	NW mainland	SE mainland	NW mainland	SE mainland
NW mainland	0.1–0.3		0.0	
SE mainland	**1.8–2.2**	0.1–1.7	**0.4–0.8**	0.2–0.6
Dinarolacerta				
(C)	*mosorensis*	*montenegrina*	*mosorensis*	*montenegrina*
mosorensis	0.1–2.6		0.2–0.4	
montenegrina	**6.9–8.4**	0.6	**2.4–2.8**	0.2
Podarcis melisellensis				
(D)*	fiumana	melisellensis	Lastovo	
fiumana	0.1–1.3			
melisellensis	**5.5–6.5**	0.1–1.3		
Lastovo	**5.9–6.4**	**4.5–5.4**	0.1–0.4	

Molecular dating

Results of the molecular dating using BEAST analysis are presented in Fig. 3. Due to the wide ranges of posterior density intervals (95% HPD), the calculated times can only be considered as rough estimates. The main splits between the lineages of *D. oxycephala* can be estimated at about 5 million years ago (mya) for the ‘island’–‘mainland’ clade separation and at about 0.8 mya for the ‘SE–NW mainland’ sublineages. The primary radiation within *D. mosorensis* may be dated at about 1 mya, while *D. mosorensis* and *D. montenegrina* diverged around 5 mya.

Discussion

Phylogeography

The phylogeographical structure of Dalmatolacerta oxycephala

The shapes of Mantel test plots of the relationship between geographical and genetic distances can discriminate between cases in which the dispersal process is affected not only by distance but also by the presence of barriers to gene flow (Guillot et al. 2009). As is the case for *Podarcis melisellensis*, discontinuous distributions of points in the scatter plot of genetic vs. geographical distance (three groups) for *D. oxycephala* suggest that the dispersal process is not affected exclusively by distances (Fig. S1), but also by some kind of barriers to gene flow. The ‘island clade’ of *D. oxycephala* co-occurs with representatives of all clades and subclades of *P. melisellensis* (Fig. 1d, Fig. S2). Podnar et al. (2004) proposed that the three main clades of *P. melisellensis* were separated some 2 mya by geographical barriers to gene flow such as sea channels or salty swamps. The very deep phylogenetic gap found between the ‘island’ and ‘mainland’ clades of *D. oxycephala* implies an old age of the lineages. Coalescent time can be dated to about 5 mya. This time corresponds to the end of the Messinian salinity crisis as well as a period of rapid uplift of the Dinaric chain, which strongly affected the palaeogeography and topography of the region (Marović et al. 1999; Kuhlemann 2003, 2007). The two main

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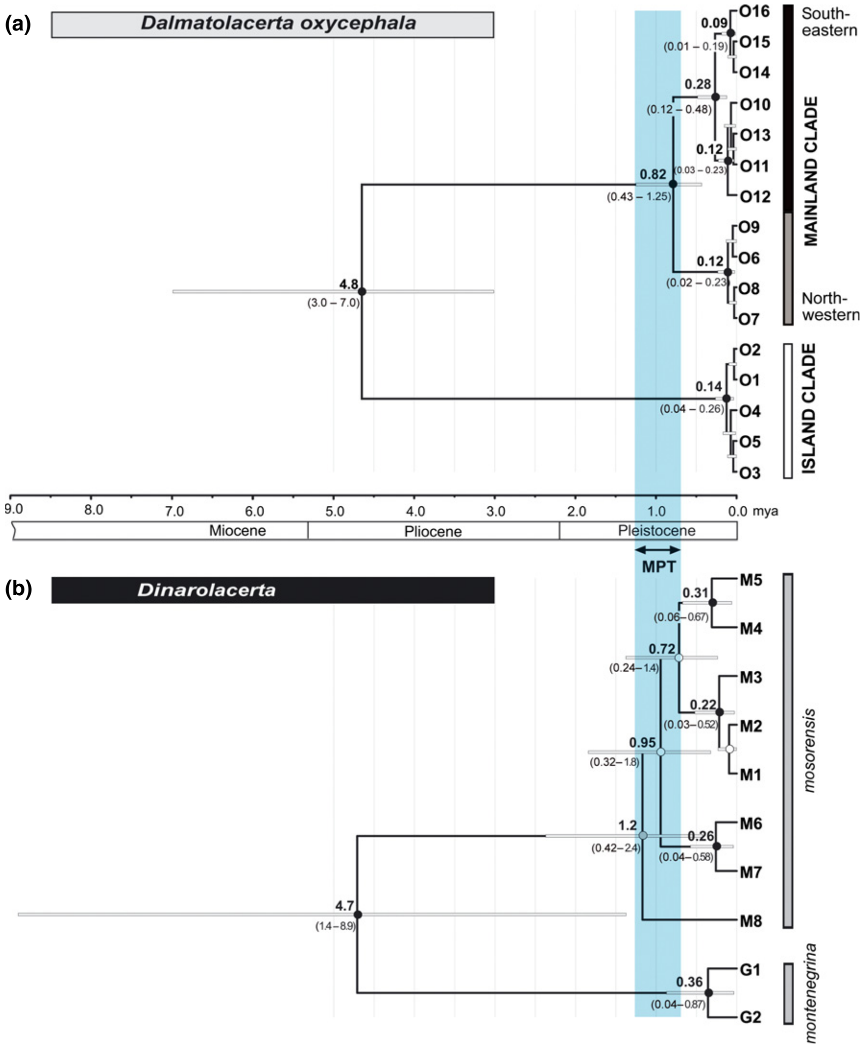

Fig. 3. Ultrametric tree based on *cyt b* data representing molecular dating of the main splits within (a) *Dalmatolacerta oxycephala* and (b) *Dinarolacerta*. Node ages (in my) are indicated by the numbers above nodes. 95% confidence intervals for estimated node ages (95% HPD) are represented by grey bars and are also given numerically in the brackets below the bars. The blue vertical bar depicts the period of Middle Pleistocene Transition (MPT). Bayesian posterior probabilities of 1, 0.9–0.99 and lower than 0.9 are indicated by black, grey and white circles, respectively.

clades may have diversified in allopatry and evolved afterwards most probably as warm (‘island clade’) and cold (‘mainland clade’) adapted forms. While the ‘island clade’ remained restricted exclusively to the area under strong Mediterranean climatic influence (southern Adriatic coast and islands), the mainland clade apparently has acquired a wider range of climatic tolerance, because it is found in Mediterranean as well as in mountainous region. Therefore, it seems that the adaptation to colder climatic conditions allowed the ‘mainland clade’ to colonize the colder Dinaric mountains, where an Alpine climate characterized by strong altitudinal differences in precipitation and temperature predominates (Reed et al. 2004).

Today, the ‘island clade’ is distributed over the remote island groups (Lastovo and Vis archipelagos), as well as in the coastal region and on the offshore islands of south Dalmatia. The Lastovo and Vis archipelagos were most probably separated from each other as well as from the mainland (including offshore islands) by sea channels or salty swamps even during the Würm sea-level low-stand (Podnar et al. 2004 and references therein). It could be expected that this kind of barrier would act even more profoundly on a rock climbing species such as *D. oxycephala*. Therefore, taking into account the very small haplotype diversity within this clade, it is very probable that its Würm refuge was located in only one region, most probably the Vis archipelago or the Lastovo archipelago. However, its precise location cannot be deduced from our data. From this refuge, the expansion may have taken place only recently by rafting or anthropogenic influence. A very plausible scenario accounts for colonization via merchandising, especially stone trade. Among Balkan lacertids, *D. oxycephala* is the species best adapted to rock climbing (Bejaković et al. 1995). It also inhabits numerous quarries on Dalmatian islands and on the coast. Quarries and stone trade have an old tradition in this area. The occurrence of *D. oxycephala* in the small town of Osor on the North Adriatic Island Cres (Tóth et al. 2006) more than 100 km away from the northern edge of its natural distribution range could possibly be explained by stone trade.

The ‘mainland clade’ inhabits almost the whole mainland area of the species and was additionally found on the island of Korčula. It is further divided into two subclades, a south-eastern one in Montenegro and Albania and a north-western one in Croatia and the Herzegovina (Fig. 1e, Fig. 2). Haplotypes of both subclades have been found syntopic in Korita, a Herzegovinian village close to the Montenegrin border (Fig 1d). The low variability of haplotypes within subclades and their geographical distribution suggest two or three relatively small glacial refugia.

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The north-western mainland subclade lacks a remarkable phylogenetic structure, indicating that this population experienced a strong bottleneck in the small glacial refugium, located probably in central Dalmatia.

As far as the south-eastern mainland subclade is concerned, two lineages separated by four mutational steps may speak in favour of the existence of two microrefugia (western and eastern) within Montenegro's refugium. Tomović et al. (2000) explained the finding of an isolated population of *D. oxycephala* at the northern slope of the Durmitor massif by the refugial character of the Tara River canyon. Thus, it can be assumed that the eastern microrefugium was located in this region. The western one was probably situated at the Lovćen Mt. foothills where great haplotype diversity within an extremely small region was observed (Fig. 1d and e). Another possibility would be that there was only one bigger refuge in the coastal region of Montenegro and that the missing haplotypes resulted from sampling error.

The phylogeographical structure of Dinarolacerta

The *D. mosorensis* lineage is characterized by a high number of quite distant haplotypes which do not form close clades. In the TCS network (Fig. 1f), the eight haplotypes form six distinct, genetically very divergent units (M1–M3, M4, M5, M6, M7 and M8, with *cyt b* p-distances up to 2.6%, Table 2). These values are similar or higher than the differences between the two subclades of the 'fiumana' clade of *P. melisellensis* (Fig. 1g) explained by two different Würm Glacial refugia (Podnar et al. 2004). Thus, we assume that *D. mosorensis* survived unfavourable climatic periods in at least six microrefugia scattered over most of today's range of the species. However, apart from deep valleys separating mountainous regions inhabited by this species today, there are no other obvious past or present geographical barriers to gene flow between them. The Neretva River separated 'northern' and 'southern fiumana' populations of *P. melisellensis* and was also reported to constitute a significant barrier to gene flow and therefore imposed geographical isolation of the lineages of Martino's vole (*Dynaromys bogdanovi*, Kryštufek et al. 2007). Ljubisavljević et al. (2007) proposed that the Neretva River represents a zoogeographical barrier between Biokovo and all other populations of *D. mosorensis*. They also speculated that the population from the type locality of the species, the Mount Mosor, would be related to Biokovo because they are not separated by any major geographical barrier. Our results confirmed their assumptions only partially. The Mosor and Biokovo haplotypes indeed cluster together, but they are separated by as much as eight mutational steps. This level of difference in the *cyt b* gene indicates longer separation.

The largest *cyt b* distance within *D. mosorensis* (2.6%) was found between the northernmost and the southernmost populations, Mosor and Lovćen, respectively (Fig. 1d and f), corresponding to an isolation time of about 1 mya (Fig. 3). It fits well with the period of the Middle Pleistocene Transition (MPT, 1.25–0.70 mya, Fig. 3b) which is characterized by intensification of glaciations (Clark 2012). The oldest differentiation between *D. mosorensis* can be dated to 1. 2 mya, corresponding to the middle Pleistocene and the time of the beginning of major climatic oscillations (Head et al. 2008). The original centre of radiation of *D. mosorensis* was presumably located somewhere in the so-called Adriatic Triangle (Fig. 1d). This region encompasses the Prokletije mountain massif and the adjacent area (the lowland of Skutari Lake) and is known to be one of the Balkan areas with the highest level of species diversity and endemism (Džukić et al. 2003; Džukić and Kalezić 2004). Depending on altitude, it encompasses a large variety of habitats, from Mediterranean over temperate to boreal (Džukić and Kalezić 2004). Also, the splitting of *D. mosorensis* and *D. montenegrina* is proposed to have taken place in this region (Ljubisavljević et al. 2007). At present, the known range of *D. montenegrina* is restricted to the Prokletije area. The small p-distance (1.5%) between *12S rRNA* sequences of *D. montenegrina* obtained in the study of Ljubisavljević et al. (2007) and the MO-13 sample analysed here (Gene Bank accession number GQ142078) clearly shows that the latter specimen also belongs to *D. montenegrina.* This confirms therefore the assumption of Petrov (2006) that this species also inhabits the Albanian side of the Prokletije mountain. The *D. mosorensis*–*D. montenegrina* split was dated by Ljubisavljević et al. (2007) to 6.9 mya on the basis of *12S rRNA*, and our results based on the *cyt b* gene point to a somewhat younger divergence of 5 mya. Ljubisavljević et al. (2007) invoked the Morača River canyon as a geomorphological and climatic barrier that caused the speciation of the *Dinarolacerta* species. Another possibility would be the uplift of the Dinarides that took place in the time period of about 5 mya (Marović et al. 1999; Kuhlemann 2003, 2007), as already mentioned for *Dalmatolacerta*.

It can be speculated that the present-day patchy distribution of *D. mosorensis*, a cold-adapted species, represents the remnant of a much wider and more continuous range during cold Pleistocene periods. In fact, the species once again occupies its interglacial refugia. Numerous missing haplotypes in the TCS network probably went extinct during the warm interglacial periods, while only those distributed close to mountains had the opportunity to survive the unfavourable warm conditions by altitudinal range shifts. Multiple expansion and contraction events have also led to the observed lack of geographical structure because in some cases the lineages from different refugia came in secondary contact (e.g. haplotype M8 is geographically closer to the haplotypes M1, M2 and M3, and in reality, M8 was the first diverging lineage and the one less closely related, Figs. 1 and 2). A very similar scenario with range contractions to microrefugia during warmer interglacials and expansions during glaciations has recently been described for another cold-adapted reptile species, *Vipera ursinii* (Ferchaud et al. 2012). As is the case with *D. mosorensis*, *V. ursinii* occurs in isolated mountainous populations. The presently highly fragmented distribution pattern reflects its restriction to small mountainous refugia also during past warm periods.

D. mosorensis probably began to spread from its southern mountainous centre of origin during the last part of the Pleistocene Calabrian stage (approximately 1800–800 kya) when the climate became colder, and continued to radiate throughout the Günz glaciations (800–600 kya) and probably, at that time, already occupied its present range. The onset of the Günz-Mindel (600–475 kya) interglacial period caused the retreat of the species into several microrefugia located on the slopes of the Dinaric mountains. During the Mindel glaciations (475–400 kya), a re-expansion of the species occurred. However, because the duration period of this glacial was shorter than the period of time available for the original radiation, in this time only the populations from the geographically proximate microrefugia completely admixed. The same scenario was probably repeated in the same microrefugia during the following interstadials. Indeed, the continuous distributions of points in the scatter plot (Fig. S1) of genetic vs. geographical distances of *D. mosorensis* suggest that isolation by distance is the underlying cause for the obtained phylogeographical structure. During glacial periods, the species continued to expand from microrefugia and populations from geographically nearer refugia most probably came in contact. However, the complete admixture of haplotypes that diverged in different refugia did not occur. The only departure from this sce-

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nario is the finding of two very divergent *D. mosorensis* haplotypes (M3 and M8) at a single location at Mt. Durmitor that differ by 1% in the *cyt b* gene. This could be explained by secondary contact of two lineages that arose in allopatry in two different microrefugia. High haplotype diversity (haplotypes M1, M2 and M3) within a very small geographical region suggests that their refuge was most probably located in this region, while the origin of the haplotype M8, which appears as the most basal one in the phylogenetic tree (Fig. 2), could not be deduced from our results.

Although both rock lizard species investigated in this study, *D. mosorensis* and *D. oxycephala,* share similar, rocky habitats and have a largely overlapping distribution range (Fig. 1a and b), their phylogeographical patterns show little concordance (Fig. S2). The IBD analysis rejected the hypothesis of global panmixia for both species. However, for *D. oxycephala*, some kind of barriers to gene flow and, for *D. mosorensis,* isolation by distance were invoked as major causes for the present phylogeographical pattern (Fig. S1). While Quaternary climatic fluctuations caused repeated cycles of range expansions of *D. mosorensis* during cold periods, and contraction during the warm ones, *D. oxycephala* as a more warm-adapted species was restricted to its microrefugia during the cold periods. Therefore, the presently fragmented range of *D. mosorensis* may coincide with its Pleistocene interglacial refugia, and in contrast, the presently more continuous range of *D. oxycephala* might be the result of a postglacial expansion out of several microrefugia.

However, it seems that the slopes and foothills of the same Dinaric mountains repeatedly served (and still serve) as interglacial refugia for *D. mosorensis* on the one side and glacial refugia for *D. oxycephala* on the other. The complex topographic structure of that region enabled altitudinal range shifts and provided numerous suitable habitats that could meet the different ecological preferences of these two rock lizard species allaying, thereby, the negative effects of the extreme Quaternary climatic changes. The very different phylogeographical structure of *P. melisellensis* especially on the mainland can best be explained by the fact that this ground-dwelling species lives in the spacious open Mediterranean dry grass and dwarf shrub heath habitats where it can easily expand its area when the climatic conditions become favourable.

Taxonomy and conservation

The genera *Dalmatolacerta* and *Dinarolacerta* show a remarkable phylogenetic structure, each bearing two deeply separated lineages (Fig. 2). In the case of *Dinarolacerta*, the two lineages correspond to two different species: *D. mosorensis* and the recently described *D. montenegrina* (Ljubisavljević et al. 2007). In contrast, the lineages within *Dalmatolacerta* are assigned at present to the same monotypic species, *D. oxycephala*. Moreover, the three main clades of *P. melisellensis* were considered as subspecies (Podnar et al. 2004). The genetic differences between *P. melisellensis subspecies* (uncorrected p-distance about 4.5–6.5% in *cyt b*) are remarkably smaller than those between the 'island' and 'mainland' clades of *D. oxycephala* (9.4–10.1% in *cyt b*, Table 2). The observed genetic distances in *cyt b* and *16S rRNA* genes within *D. oxycephala* exceed even those found between *D. mosorensis* and the recently described *D. montenegrina* (Table 2). A comparable level of divergence in the *cyt b* gene was reported also between some closely related lacertid species, namely *Podarcis pityusensis* and *P. lilfordi*, with an average interspecific uncorrected distance in *cyt b* of 9.7% (Terrasa et al. 2004).

The cryptic diversity within *D. oxycephala* characterized by a high level of divergence between 'island' and 'mainland' lineages revealed in this study must prompt a taxonomic revision of the species.

Nuclear loci can provide independent estimates of phylogenetic relationships to corroborate (e.g. Engleder et al. 2013) or refute phylogenies constructed using mtDNA sequence data (Pinho et al. 2008; Renoult et al. 2009). Since this study is based solely on two mtDNA markers, molecular analysis of nuclear genes as well as critical morphological analysis should be performed to clarify the taxonomic status of the two main clades of *D. oxycephala*. Additionally, taking into account the continuous distribution of the species along the Adriatic coast in South Dalmatia (from Cavtat to the Neretva River delta), as well as in the Neretva canyon (Bischoff 1984), it is of great interest to locate the contact zones of the two lineages. We hope that future analyses of multilocus nuclear markers from such populations will provide information about possible hybridization between 'island' and 'mainland' lineages to decide whether they should be regarded as separate species in the light of the biological species concept.

Nevertheless, regardless of their taxonomic status (subspecific or specific), the conservation status of *D. oxycephala* should be revised because it encompasses at least two highly divergent, evolutionary significant units (ESUs) for conservation. As far as the black '*tomasinii*' is concerned, individuals belonging to this form (samples from Lovćen and Korita) clustered within both mainland subclades together with grey-coloured *D. oxycephala* s. str. (Fig. 1e). Specifically, the black- and grey-coloured individuals encompassed within the south-eastern subclade of the 'mainland' clade are separated by only two, and those within the north-western subclade are separated by a single mutational step (Fig. 1e, Fig. 2) and therefore certainly do not represent long isolated, independently evolving lineages that would justify their recognition as different taxa. It seems that in both 'mainland' subclades of *D. oxycephala*, the melanistic '*tomasinii*' morph evolved independently quite recently in both subclades, most probably as ecological adaptations to mountainous conditions. Melanism in lizards is often explained as an adaptation to condition of low solar radiation and environmental temperatures (i.e. Clusella-Trullas et al. 2007), but recently the association of melanism with high incidence of fog and cloud cover was reported for cordylid lizards (Janse van Rensburg et al. 2009). Both phenomena can be invoked as well for melanism in '*tomasinii*'.

Conclusion

In conclusion, the study revealed highly non-concordant phylogeographical patterns for the three investigated lacertid lizard species in spite of their largely sympatric distribution. These results can be explained in the light of different responses to Pleistocene climatic oscillations due to specific ecological and habitat preferences as well as the impact of past geotectonic events. For all three species, the existence of several possible microrefugia is implicated. For *P. melisellensis* and *D. oxycephala*, we assume glacial refugia, while for *D. mosorensis*, interglacial microrefugia appear more likely. Cryptic genetic diversity found within *D. oxycephala* necessitates a detailed revision of its taxonomic and conservation status.

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Supporting Information

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

Figure S1. Isolation-by-distance (IBD) scatter plots of (a) *Dalmatolacerta oxycephala*, (b) *Dinarolacerta mosorensis* and (c) *Podarcis melisellensis*.

Figure S2. TCS networks showing the lack of correlation in phylogeographical patterns of (a) *Podarcis melisellensis*, (b) *Dalmatolacerta oxycephala and* (c) *Dinarolacerta mosorensis*. Different colours correspond to four geographical regions inhabited by *P. melisellensis* (see figure legend). Haplotypes of *D. oxycephala* and *D. mosorensis* found in the overlapping zones are coloured accordingly.

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