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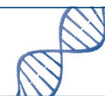

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Genetic differentiation of populations of the threatened saproxylic beetle Rosalia longicorn, *Rosalia alpina* (Coleoptera: Cerambycidae) in Central and South-east Europe

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Knowledge of patterns of genetic diversity in populations of threatened species is vital for their effective conservation. Rosalia longicorn (*Rosalia alpina*) is an endangered and strictly protected beetle. Despite a marked decline in part of its range, the beetle has recently expanded to the lowlands of Central Europe. To facilitate a better understanding of the species' biology, recent expansion and more effective conservation measures, we investigated patterns of genetic structure among 32 populations across Central and South-east Europe. Eight microsatellite loci and a partial mitochondrial gene (cytochrome *c* oxidase subunit I) were used as markers. Both markers showed a significant decline in genetic diversity with latitude, suggesting a glacial refugium in north-western Greece. The cluster analysis of the nuclear marker indicated the existence of two genetically distinct lineages meeting near the border between the Western and Eastern Carpathians. By contrast, one widespread mtDNA haplotype was dominant in most populations, leading to the assumption that a rapid expansion of a single lineage occurred across the study area. The genetic differentiation among populations from the north-western part of the study area was, however, surprisingly low. They lacked any substructure and isolation-by-distance on a scale of up to 600 km. This result suggests a strong dispersal capacity of the species, as well as a lack of migration barriers throughout the study area. That the lowland populations are closely related to those from the nearby mountains indicates repeated colonization of the lowlands. Our results further suggest that *R. alpina* mostly lives in large, open populations. Large-scale conservation measures need to be applied to allow for its continued existence. © 2015 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2015, **00**, 000–000.

ADDITIONAL KEYWORDS: beech – conservation – Natura 2000 – phylogeography – post-glacial recolonization – xylophagous insect.

INTRODUCTION

An understanding of the patterns of genetic diversity in populations of threatened species enables the development of wide-scale conservation strategies and the use of management actions according to the current needs (Avise *et al.*, 1987; Moritz, 1994). Population genetic structure is determined by genetic isolation, which is governed by the forces of genetic drift, natural selection, and gene flow (Slatkin,

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1987). Furthermore, restrictions in the landscape permeability can reduce gene flow between habitat patches and decrease the effective population size. Such populations are then more prone to the effects of genetic drift that decrease genetic diversity and inhibit adaptability to a changing environment (Frankham, Ballou & Briscoe, 2002). Simple conservation of species that neglects to consider its population structure may thus fail to address isolated and genetically eroded populations or, by contrast, reveal the reservoirs of genetic diversity such as glacial refugia. Consequently, a broad range of molecular techniques have been used to understand the nature of population structure, and they become an important tool in many studies of threatened and protected species (Sunnucks, 2000; Morin, Luikart & Wayne, 2004; Behura, 2006).

Saproxylic insects are a diverse group with high ecological and economical importance (Grove, 2002). Genetic structure has occasionally been studied in the important pest species (Horn *et al.*, 2006, 2009; Sallé *et al.*, 2007; Carter, Smith & Harrison, 2010). The saproxylic guild, however, also contains a number of threatened species, and some of them serve as models for ecology, conservation biology, and/or as umbrella species (Ranius, 2002; Buse, Schroder & Assmann, 2007). Despite that, very little is known about their population genetics, and this may compromise conservation efforts; but see also Cox *et al.* (2013); Oleksa *et al.* (2013); Solano *et al.* (2013); Drag & Cizek (2014); Oleksa *et al.* (2015).

The Rosalia longicorn (*Rosalia alpina*; Linnaeus, 1758) is an endangered and strictly protected saproxylic beetle. It is listed as a priority species under the European Union (EU) Habitats Directive, which makes it an icon of invertebrate conservation in Europe. Its distribution range covers most of Europe; from the Pyrenees, the Alps, and the Carpathians, to Crimea, the Caucasus, and the Urals (Sama, 2002). In the south, the beetle reaches Corsica, Sicily, Greece, and the Turkish province of Hatay. In the north, the species has experienced substantial retreat because it has disappeared from Scandinavia, most of Germany, Poland, and Czech Republic (Sláma, 1998; Lindhe, Jeppsson & Ehnström, 2011; Michalcewicz & Ciach, 2015). Despite a notable decline, the distribution of the species is rather continuous in two large mountain systems: in the Alps and the Carpathians, as well as on the Balkan Peninsula (Sláma, 1998; Gepp, 2002; Duelli & Wermelinger, 2005).

Although generally considered a montane species associated with European beech *Fagus sylvatica* L. (Heyrovský, 1955; Sláma, 1998), *R. alpina* also inhabits lowlands and utilizes a wide range of broadleaved trees. The lowland populations were for long known to occur in Western (Picard, 1929) and Southeastern (Serafim & Maincan, 2008) Europe. Recently, they have also been repeatedly reported in Central Europe, mostly from the floodplains of the Danube and its tributaries (Jendek & Jendek, 2006; Cizek *et al.*, 2009; Hovorka, 2011). As a result of a lack of earlier records, it has been proposed that the beetle spread to the lowlands of Central Europe only recently (Cizek *et al.*, 2009). Such a sudden expansion into the previously unexploited habitat accompanied by a switch in the host plant may indicate the existence of distinct ecotypes of the species, associated with upland and lowland forests or beech and other hosts.

Two scenarios for the colonization of Central European lowlands were proposed, including downslope colonization by nearby upland populations and colonization by a lowland population from elsewhere (Cizek *et al.*, 2009). A shift of upland populations to lowlands would either require a change in the species' host and/or habitat preference or in the quality of the newly-colonized habitat. Colonization by a lowland population originating outside the region would suggest the existence of a lowland lineage, adapted to different habitats and hosts. The host-associated population structure, in which populations exploiting different resources are genetically distinct (Stireman, Nason & Heard, 2005; Ferrari *et al.*, 2012), is well documented in phytophagous insects and demonstrates how the environment can impact gene flow, even in the absence of physical barriers (Feder *et al.*, 1994; Via, Bouck & Skillman, 2000).

In the present study, we analyzed parts of nuclear (microsatellites) and mitochondrial (cytochrome *c* oxidase subunit I; COI) DNA of > 30 populations of *R. alpina* from Central and South-east Europe aiming to identify the patterns of their genetic diversity and phylogeographical structure. A broad range of the statistical methods were applied to test hypotheses about presumed refugia and species history. By comparing the genetic structure of populations from lowlands and nearby mountains, we attempted to reveal the relationship between populations originating from different habitats. Information on patterns of genetic diversity in *R. alpina* populations may increase our knowledge of the species' biology and facilitate more effective conservation.

MATERIAL AND METHODS

Sample collection and DNA extraction

The material analyzed included beetles from 33 localities in Central and South-east Europe, thus covering a significant part of the species' distribution in

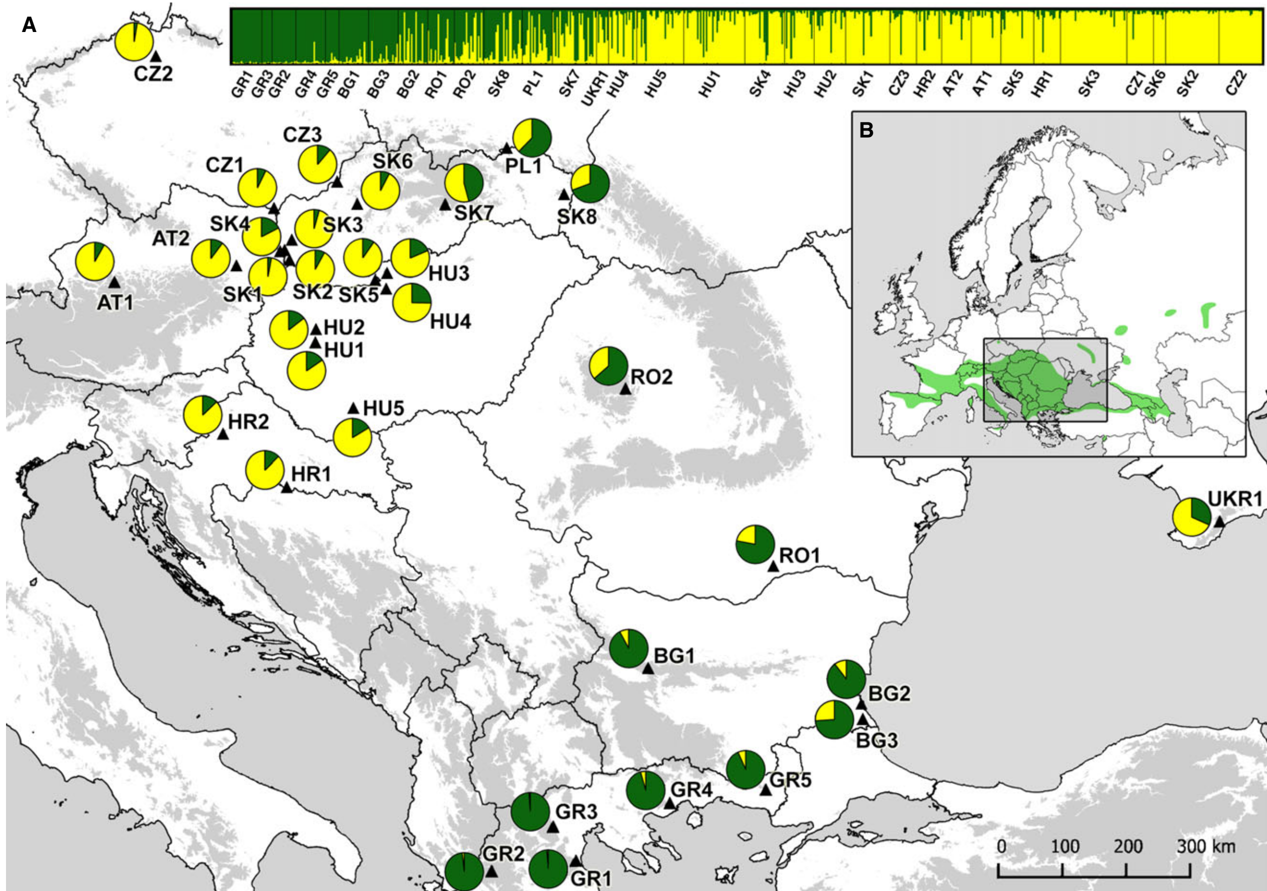

Figure 1. A, localization and the genetic structure of 32 populations (full names are listed in Table 1) of the Rosalia longicorn (*Rosalia alpina*) based on eight microsatellite loci. Each pie chart represents a proportion of membership of individuals from a given population in each of the two clusters indicated by the Bayesian clustering analysis (STRUCTURE). Grey surface represents the area above 800 m a.s.l. Upper barplot illustrates the division of each individual into two colours, reflecting the estimated assignment into two clusters. Solid black lines define the boundaries between the same populations as in the map. B, distribution range of *R. alpina* (light green colour) and the study area (black rectangle).

Europe (Fig. 1B). Samples originated from both the uplands (altitudinal range 300–1050 m a.s.l.; 27 populations) and the lowlands (0–300 m a.s.l.; seven populations). The upland sites were dominated by beech (*Fagus* spp.) forests, and the beetles were collected on beech wood. In the lowlands, the beetles were always associated with other tree species, including elms (*Ulmus* spp.), maples (*Acer* spp.), and ash (*Fraxinus* spp.). Details on the localities, host plants, and number of individuals analyzed are provided in Table 1. A part of a middle leg from all discovered specimens (dead or alive) was taken and stored in vials containing 96% ethanol for molecular analyses. Genomic DNA was extracted from the sampled tissue using the Genomic DNA Mini Kit Tissue (Geneaid) in accordance with the manufacturer's instructions.

MICROSATELLITES

Amplification

In total, 700 individuals were genotyped for nine polymorphic microsatellite loci previously described by Drag, Zima & Cizek (2013). Because of an insufficient sample size, one population was excluded from the microsatellite's dataset, thus leaving a total of 32 populations and 695 individuals. The locus RA_29 repeatedly failed to amplify in many individuals of some populations. We thus decided to exclude it from further analyses. Hence, all of the reported results in the presents study are based on eight loci. Polymerase chain reaction (PCR) products were analyzed with an automated sequencer ABI 3730XL (Applied Biosystems) by a commercial company (Macrogen Inc.). Allelic patterns were scored using GENEMAPPER, version 3.7 (Applied Biosystems).

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Table 1. Sampled populations of the Rosalia longicorn (*Rosalia alpina*) with information about their habitat, host tree, GPS coordinates, and the number of individuals analyzed for eight microsatellite loci and cytochrome c oxidase I (COI)

							Number of individuals analyzed	
Country	Locality	Code	Habitat	Host tree	Latitude	Longitude	COI	Microsatellites
Austria	Kalkalpen	AT1	U	FS	47.805571	13.950015	5	20
Austria	Wienerwald	AT2	U	FS	48.010122	16.199364	5	20
Bulgaria	Stara planina II	BG1	U	FS	42.784421	23.790154	5	20
Bulgaria	Strandja	BG2	U	FO	42.08869	27.750227	5	20
Bulgaria	Ropotamo	BG3	L	AC, FA	42.297936	27.724085	5	20
Czech Republic	Dyje floodplain	CZ1	L	AC, UL	48.718113	16.892834	5	18
Czech Republic	Bezdez	CZ2	U	FS	50.539185	14.720318	5	29
Czech Republic	Bile Karpaty	CZ3	U	FS	49.032422	18.025136	5	18
Greece	Olymp	GR1	U	FS	40.108456	22.460764	5	20
Greece	Pindos	GR2	U	FS	39.959741	20.906086	5	16
Greece	Vermio	GR3	U	FS	40.589685	22.042605	5	7
Greece	Rodopi	GR4	U	FS	40.921661	24.189622	5	20
Greece	Evros	GR5	U	FS	41.109798	25.962128	5	9
Croatia	Lonsko Polje	HR1	L	FA	45.196004	17.128523	5	18
Croatia	Mt. Medvednica	HR2	U	FS	45.88291	15.953268	5	17
Hungary	Kab-Hegy	HU1	U	FS	47.04975	17.655917	5	41
Hungary	Bakony	HU2	U	FS	47.215248	17.666995	5	21
Hungary	Borzsony	HU3	U	FS	47.917322	18.977368	5	20
Hungary	Pilis	HU4	U	FS	47.721766	18.960977	5	19
Hungary	Mecsek	HU5	U	FS	46.21439	18.355765	5	32
Poland	Beskid Nisky	PL1	U	FS	49.446583	21.184611	5	20
Romania	Comana forest	RO1	L	AC, T, F	44.15751	26.100216	5	18
Romania	Apuseni	RO2	U	FS	46.461244	23.374803	5	20
Slovakia	Bratislava	SK1	U	FS	48.203872	17.0931	5	30
Slovakia	Dunajské Luhy	SK2	L	AC, AP, UL	48.080593	17.179173	5	36
Slovakia	Male Karpaty	SK3	U	FS	48.327994	17.220819	5	45
Slovakia	Devinska Kobyla	SK4	L	FE	48.186665	17.005319	5	27
Slovakia	Kovacov	SK5	L	Q	47.827912	18.75877	5	22
Slovakia	Strazovske vrchy	SK6	U	FS	48.768333	18.427567	5	8
Slovakia	Muranska planina	SK7	U	FS	48.760975	20.050352	4	30
Slovakia	Vihorlat	SK8	U	FS	48.886894	22.242261	5	26
Serbia	Stara planina	SR1	U	FS	44.173172	22.123664	5	0
Ukraine	Crimea	UKR1	U	FO	44.74788	34.333431	5	8

U, uplands; L, lowlands; FS, *Fagus sylvatica*; FO, *Fagus orientalis*; AC, *Acer campestre*; AP, *Acer pseudoplatanus*; UL, *Ulmus laevis*; FA, *Fraxinus angustifolia*; FE, *Fraxinus excelsior*; F, *Fraxinus* sp.; T, *Tilia* sp.; Q, *Quercus* sp.

Loci characteristics and genetic diversity

Frequencies of null alleles were estimated using FREENA (Chapuis & Estoup, 2007). The linkage disequilibrium between all pairs of loci, as well as the Hardy–Weinberg equilibrium (HWE) across loci and populations, was tested with GENEPOP, version 4.1.3 (Raymond & Rousset, 1995; Rousset, 2008) using the default parameters.

For each population, we calculated the number of alleles (N_A), observed (H_O) and expected (H_E) heterozygosity, and the number of private alleles (N_{PA}) using GENALEX, version 6.5 (Peakall &

Smouse, 2006, 2012). Allelic richness (AR) corrected for sample size variability was computed in FSTAT, version 2.9.3.2 (Goudet, 2002). Population specific coefficient of inbreeding (F_{IS}) was calculated and tested using ARLEQUIN, version 3.5.1.3 (Excoffier & Lischer, 2010) with 10 000 permutations. Two indices (H_E and AR), which represented the genetic diversity of populations, were used in a linear regression as a function of latitude and longitude.

Population structure

The population structure of our dataset was assessed using STRUCTURE, version 2.3.4 (Pritchard, Stephens & Donnelly, 2000), assuming an admixture model (individuals may have mixed ancestry) and correlated allele frequencies (closely-related populations might have correlated allele frequencies). We ran STRUCTURE for values of K ranging from 1 to 10 with 100 000 burn-in and 1 000 000 Markov chain Monte Carlo (MCMC) steps for 10 replicates for each K. The best K value was chosen according to Evanno, Regnaut & Goudet (2005), using the STRUCTURE HARVESTER (Earl & vonHoldt, 2012). The results obtained for a given K were post-processed in CLUMPP, version 1.1.2 (Jakobsson & Rosenberg, 2007) and used to generate pie charts, illustrating the geographical structure of each population. Barplots were visualized in DISTRUCT, version 1.1 (Rosenberg, 2004).

Genetic differentiation

All populations were assigned into two groups according to their habitat ('lowland' and 'upland') (Table 1). Genetic differentiation among populations within and between the two groups was tested using analysis of molecular variance (AMOVA) in ARLEQUIN, version 3.5.1.3 (Excoffier & Lischer, 2010) with 10 000 permutations.

To construct the phylogenetic tree of populations, we performed the evolutionary analysis of allele frequencies using a neighbour-joining (NJ) method in POPTREE2 (Takezaki, Nei & Tamura, 2010) with 10 000 bootstrap replicates. As genetic measures, we used D_A distance values (Nei & Chesser, 1983). Furthermore, alternate indices based on D_{ST} distance or F_{ST} produced congruent results (data not shown). Finally, we adjusted the constructed NJ tree in MEGA, version 6 (Tamura *et al.*, 2013).

Gene flow

The pairwise geographical distances between all populations were computed from the list of coordinates using the GEOGRAPHIC DISTANCE MATRIX GENERATOR, version 1.2.3 (Ersts, 2015). Their logarithmic values (log) were plotted against the linearized form of pairwise genetic distances $F_{ST}/(1 - F_{ST})$. The significance of correlation was tested by a Mantel test (Mantel, 1967) using the IBDWS (Jensen, Bohonak & Kelley, 2005) with 10 000 permutations. IBDWS calculates the slope and intercept of the isolation-by-distance (IBD) relationship using reduced major axis regression, which is more appropriate than standard linear regression (Bohonak, 2002). IBD patterns were calculated for all populations as well as within the north-western (NW) and south-eastern (SE) lineages gained from STRUCTURE. The UKR1 population was omitted from the IBD analyses as a result of the low number of samples available for this highly remote and isolated population.

The gene flow among populations was estimated as pairwise F_{ST} values calculated in ARLEQUIN, version 3.5.1.3 (Excoffier & Lischer, 2010). The significance of the derived genetic distances was tested by 10 000 permutations. To avoid any potential bias by null alleles, we also calculated the pairwise F_{ST} values using a correction for null alleles method implemented in FREENA (Chapuis & Estoup, 2007).

COI

Amplification

A partial fragment of the mitochondrial gene for COI (approximately 766 bp in length) was amplified and sequenced for 164 individuals from the 33 populations. Five individuals represented each population, with the exception of SK7, which was composed of four individuals. We used universal forward and reverse primers: C1-J-2183 (alias Jerry) (5′-CAA CAT TTA TTT TGA TTT TTT GG-3′) and TL2-N-3014 (alias Pat) (5′-TTC AAT GCA CTT ATT CTG CCA TAT TA-3′) (Simon *et al.*, 1994). PCR cycling parameters included a denaturation step at 95 °C for 5 min and 40 cycles at: 92 °C for 30 s, 50 °C for 30 s, and 72 °C for 1 min 30 s. The cycling concluded with a final elongation step at 72 °C for 10 min. Sequencing was performed by a commercial company (Macrogen Inc.).

Genetic diversity and differentiation

The sequences from each individual were edited and aligned (MUSCLE, default settings) in GENEIOUS, version 6.1.6 (Biomatters). To minimize the probability of the occurrence of nuclear mitochondrial pseudogenes in our data, we carefully checked all sequences for the presence of double peaks, indels, frameshifts, and stop codons, as suggested by Song *et al.* (2008). Although such measures cannot completely rule out their presence, they can minimize any possibility and also prevent overestimation of the genetic diversity indices and misinterpretation of the phylogeography (Haran *et al.*, 2015). All unique sequences were

submitted to GenBank (accession number: KT351997–KT352027). For each population, standard genetic indices such as the number of haplotypes (H), the haplotype (h) and nucleotide (π) diversities, and the number of polymorphic sites (P) were computed using DNASP, version 5.10 (Librado & Rozas, 2009). Two indices (h and π) representing the genetic diversity of populations (similar to those used for microsatellites) were used in a linear regression. A haplotype network was produced using the statistical parsimony method (95% connection limit; Templeton, Crandall & Sing, 1992), implemented in TCS, version 1.21 (Clement, Posada & Crandall, 2000). The genetic differentiation among populations inhabiting different habitats ('lowland' and 'upland') was estimated using AMOVA in ARLEQUIN, version 3.5.1.3 (Excoffier & Lischer, 2010) with 10 000 permutations.

RESULTS

MICROSATELLITES

Loci characteristics and genetic diversity

In total, 695 individuals from 32 populations were genotyped at eight microsatellite loci. All analyzed loci were polymorphic, with the number of alleles per locus ranging from five to sixteen (mean 8.25). With the exception of RA_37 paired with: RA_11 (HU5; $P = 0.00404$), RA_08 (RO2; $P = 0.00359$), and RA_13 (SK8; $P = 0.00333$), no linkage disequilibrium was found between all pairs of loci for each population after Bonferroni correction for multiple tests. The mean estimated frequency of null alleles per locus across all populations was never higher than 15%. However, in some populations and some loci, the presence of null alleles exceeded such probability (see Supporting information, Table S1).

Populations with the highest genetic diversity were found in Greece, with the mean number of alleles per locus ranging from 3.1 to 4.8. The lowest diversity was observed in UKR1 (1.8), CZ2 (2.3), and CZ1 (2.3) (Table 2). We found the same pattern for allelic richness (AR) when we considered the variation in population sizes. The mean observed and expected heterozygosity of all loci ranged from 0.172 to 0.602, and from 0.167 to 0.663, respectively. We found deviations from HWE for 17 populations (after Bonferroni correction), with the maximum of three deviating loci per population. Private alleles were recorded for twelve populations, with the frequencies ranging from 0.011 to 0.094. We found a significant decrease in genetic diversity with increasing latitude for both H_E ($R = 0.397$, $P < 0.05$) and AR ($R = 0.659$, $P < 0.05$). On the other hand, we did not find any evidence for a change in H_E or AR on the longitudinal gradient.

Population structure

According to ΔK (Evanno *et al.*, 2005), the best number of clusters was identified as two ($K = 2$) in STRUCTURE (see Supporting information, Fig. S1). It divided populations from the study area into the NW and SE lineages (Fig. 1A). The boundary between the two lineages was not well defined, and nearby populations represented a mixture of these two clusters. We were unable to detect further substructure when $K > 2$, except by the clear separation of three Greek populations (GR1, GR2, GR3) for $K \geq 4$ (see Supporting information, Fig. S1).

Genetic differentiation

AMOVA showed no genetic structure between the 'lowland' and 'upland' habitat groups (−0.6%, $F_{CT} = -0.0055$, $P = 0.633$), and little variation among populations within these groups (12.1%, $F_{SC} = 0.1205$, $P < 0.0001$). Most of the variation in the microsatellites might be explained by variation within populations (88.4%, $F_{ST} = 0.1156$, $P < 0.0001$).

The NJ tree, based on D_A distances among populations was congruent with the results gained from STRUCTURE. Although the bootstrap supports of some branches were rather weak, there was an apparent division between the populations inhabiting NW and SE parts of the study area (Fig. 2).

Gene flow

Genetic and geographical distances were correlated among all populations ($r = 0.632$, Mantel test: $P < 0.001$), as well as among populations belonging to the SE lineage ($r = 0.406$, Mantel test: $P = 0.016$) (Fig. 3). Within the NW lineage, however, we found no relationship between geographical and genetic distances ($r = 0.193$, Mantel test: $P = 0.092$), despite the fact that the longest geographical distance between two populations was more than 620 km.

F_{ST} values were significantly different ($P < 0.05$) in 88.3% of all pairwise comparisons. They showed a rather variable amount of genetic differentiation ranging from values close to zero (many pairs of populations in the northern area) to 0.44 (between GR3 and UKR1) (see Supporting information, Table S2). We achieved similar results after the ENA (= excluding null alleles) correction (Chapuis & Estoup, 2007), with the highest value of $F_{ST} = 0.43$ between GR3 and UKR1.

COI

Genetic diversity and differentiation

We identified 31 different haplotypes based on the 766-bp long fragment of the mitochondrial gene COI. The overall haplotype diversity (h) was 0.541 and the nucleotide diversity (π) was 0.0016 (Table 2). The highest diversity values (both haplotype and

Table 2. Genetic diversities of the sampled populations of the Rosalia longicorn (*Rosalia alpina*) derived from eight microsatellite loci and cytochrome *c* oxidase I (COI)

	Microsatellites						COI			
Population	N_A	AR	H_O	H_E	N_{PA}	F_{IS}	H	h	π	P
AT1†	2.6	2.38	0.378	0.458	0	0.194**	1	0	0	0
AT2	2.8	2.57	0.404	0.473	0	0.164*	3	0.7	0.00104	2
BG1†	3.0	2.50	0.320	0.450	0	0.307***	2	0.4	0.00104	2
BG2 †	3.3	2.64	0.277	0.408	0	0.322***	2	0.4	0.00052	1
BG3†	3.4	2.73	0.286	0.434	2	0.354***	1	0	0	0
CZ1	2.3	2.11	0.333	0.362	0	0.108	1	0	0	0
CZ2	2.3	2.00	0.297	0.318	0	0.083	1	0	0	0
CZ3	2.9	2.55	0.395	0.454	0	0.140*	1	0	0	0
GR1†	4.8	3.84	0.456	0.585	2	0.245***	4	0.9	0.00468	8
GR2†	4.6	4.06	0.602	0.663	1	0.124*	5	1	0.00649	10
GR3	3.6	3.63	0.500	0.560	0	0.182*	4	0.9	0.0026	4
GR4†	3.6	2.83	0.338	0.440	1	0.256***	2	0.4	0.00208	4
GR5	3.1	3.01	0.375	0.511	0	0.320**	2	0.4	0.00052	1
HR1	2.6	2.47	0.465	0.464	0	0.025	1	0	0	0
HR2	2.9	2.53	0.347	0.429	0	0.210**	2	0.4	0.00052	1
HU1†	3.0	2.48	0.442	0.445	0	0.019	2	0.4	0.00052	1
HU2†	3.0	2.57	0.369	0.445	1	0.194**	1	0	0	0
HU3†	2.9	2.62	0.394	0.456	1	0.162*	2	0.4	0.00052	1
HU4†	3.0	2.66	0.349	0.463	0	0.272***	2	0.4	0.00052	1
HU5	2.9	2.49	0.414	0.464	1	0.123*	3	0.7	0.00104	2
PL1†	3.0	2.72	0.438	0.491	0	0.135*	2	0.6	0.00078	1
RO1†	3.4	2.93	0.375	0.545	0	0.314***	2	0.6	0.00078	1
RO2	3.0	2.57	0.413	0.454	1	0.116	3	0.8	0.00156	2
SK1†	3.0	2.41	0.375	0.443	0	0.170**	2	0	0	1
SK2†	2.8	2.23	0.345	0.414	1	0.161**	2	0.4	0.00052	1
SK3	3.3	2.43	0.372	0.434	1	0.154**	1	0	0	0
SK4†	3.1	2.64	0.435	0.491	0	0.123*	1	0	0	0
SK5	3.0	2.54	0.392	0.432	0	0.115	2	0.4	0.00052	1
SK6	2.6	2.58	0.453	0.442	0	0.042	2	0.4	0.00052	1
SK7†	3.5	2.77	0.363	0.478	1	0.258***	2	0.5	0.00065	1
SK8	3.3	2.61	0.428	0.450	2	0.069	3	0.8	0.0013	2
SR1							2	0.4	0.00052	1
UKR1	1.8	1.70	0.172	0.167	0	0.038	2	0.4	0.00052	1

N_A, mean number of alleles across the eight loci; *AR*, mean allelic richness per locus, based on a minimum of seven individuals; H_O, observed heterozygosities; H_E, expected heterozygosities; N_{PA}, number of private alleles; F_{IS}, genetic similarity of individuals within the population (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$); H, number of haplotypes; h, haplotype diversity; π, nucleotide diversity; P, number of polymorphic sites.
†Significant deviation from Hardy–Weinberg equilibrium.

nucleotide) were found in the three Greek populations (GR1, GR2, GR3). As for microsatellites, we observed a significant increase in haplotype ($R = 0.42$, $P < 0.05$) and nucleotide ($R = 0.621$, $P < 0.05$) diversities with decreasing latitude, although no evidence for change in either parameters along the longitudinal gradient.

The haplotype network based on the statistical parsimony analysis showed one dominating haplotype widely present in many of the studied populations (H1; 67% of all individuals), one haplotype frequently present, especially in the Carpathian Mountains (H3; 10% of all individuals), and many rare haplotypes that were separated by one or two mutations from the H1 and often unique for a given population (Fig. 4). Beside this star-like pattern, all haplotypes of the three Greek populations (GR1, GR2, and GR3) formed a separate and more diverse network, where the most common haplotype (H1) was missing. Nevertheless, even the most remote Greek haplotype was not separated by more than seven mutations from the central haplotype.

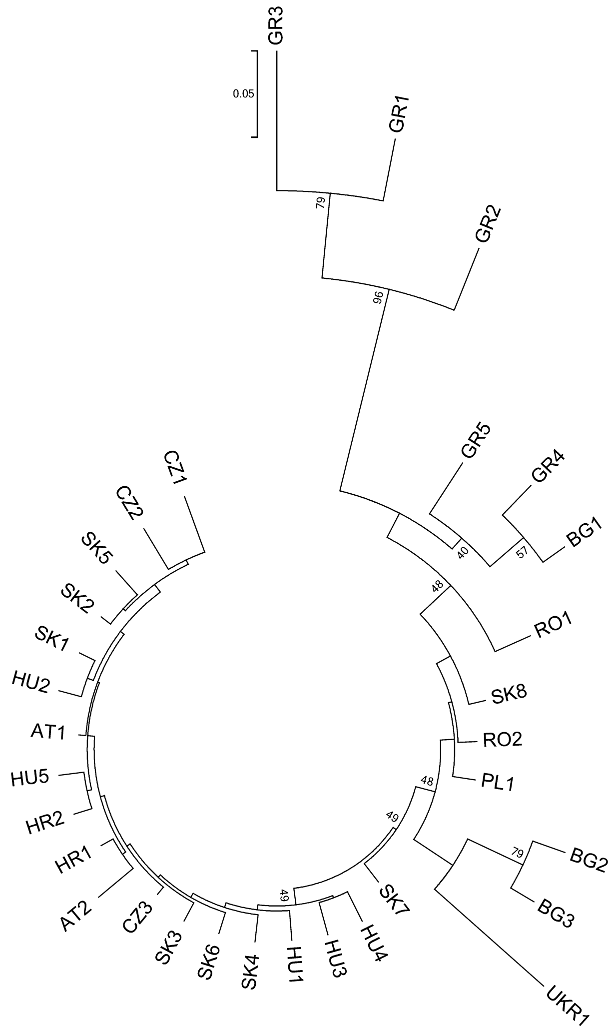

Figure 2. Unrooted neighbour-joining tree of 32 populations of the Rosalia longicorn (*Rosalia alpina*) based on D_A distance values from eight microsatellite loci. Bootstrap values above 40% are shown (10 000 replicates). Full names of the locations are listed in Table 1.

Similar to the microsatellite data, AMOVA showed no genetic structure between the 'lowland' and 'upland' habitat groups (3.1%; F_{CT} = 0.03114, P = 0.116) and most of the variation was found within populations (60.4%; F_{ST} = 0.1156, P < 0.0001). On the other hand, variation among populations within habitat groups was substantially higher than for microsatellites (36.5%; F_{SC} = 0.3766, P < 0.0001).

DISCUSSION

The present study represents the first analysis of spatial genetic variations of the endangered Rosalia longicorn beetle, *R. alpina*. We present an analysis of populations from a substantial part of the beetle's distributional range using both nuclear and mitochondrial genetic markers. Our results investigate patterns of genetic diversity of this EU-wide protected species and provide new information about the beetle's population biology.

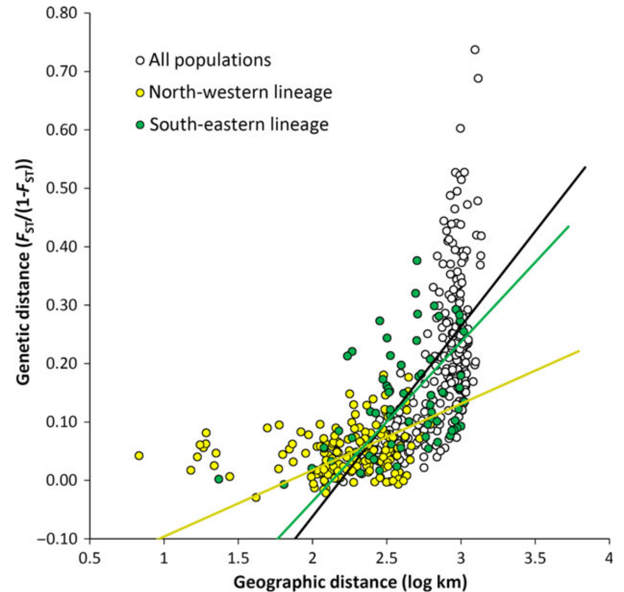

Figure 3. Isolation-by-distance (IBD) analysis of Rosalia longicorn (*Rosalia alpina*) species. Genetic differentiation (estimated as $F_{ST}/1 - F_{ST}$ based on the eight microsatellite loci) was plotted against logarithm of geographical distances (km). IBD was constructed across 31 populations (r = 0.632, Mantel test: P < 0.001), within the north-western lineage (r = 0.193, Mantel test: P = 0.092), and within the south-eastern lineage (r = 0.406, Mantel test: P = 0.016). Population UKR1 was excluded.

Phylogeography and population structure

We observed a significant decline in genetic diversity of both markers with latitude. This is most likely a consequence of the gradual loss of genetic variation with increasing distance from the glacial refugium. This form of 'southern richness and northern purity' is a common pattern among temperate species caused by post-glacial colonization (Hewitt, 1999) and frequently found in many taxons (Comps *et al.*, 2001; Gassert *et al.*, 2013; Wielstra *et al.*, 2013; Tison *et al.*, 2014). Thus, rather unsurprisingly for a beetle that depends on broadleaved trees, *R. alpina* is among the species that expanded during the warm periods of the Pleistocene cyclic climate changes. It means that *R. alpina* is not technically an 'alpine' species (*sensu* Schmitt, 2007).

The exceptional high AR and haplotype diversity of the southern populations (especially of three Greek populations GR1, GR2, and GR3) suggests the

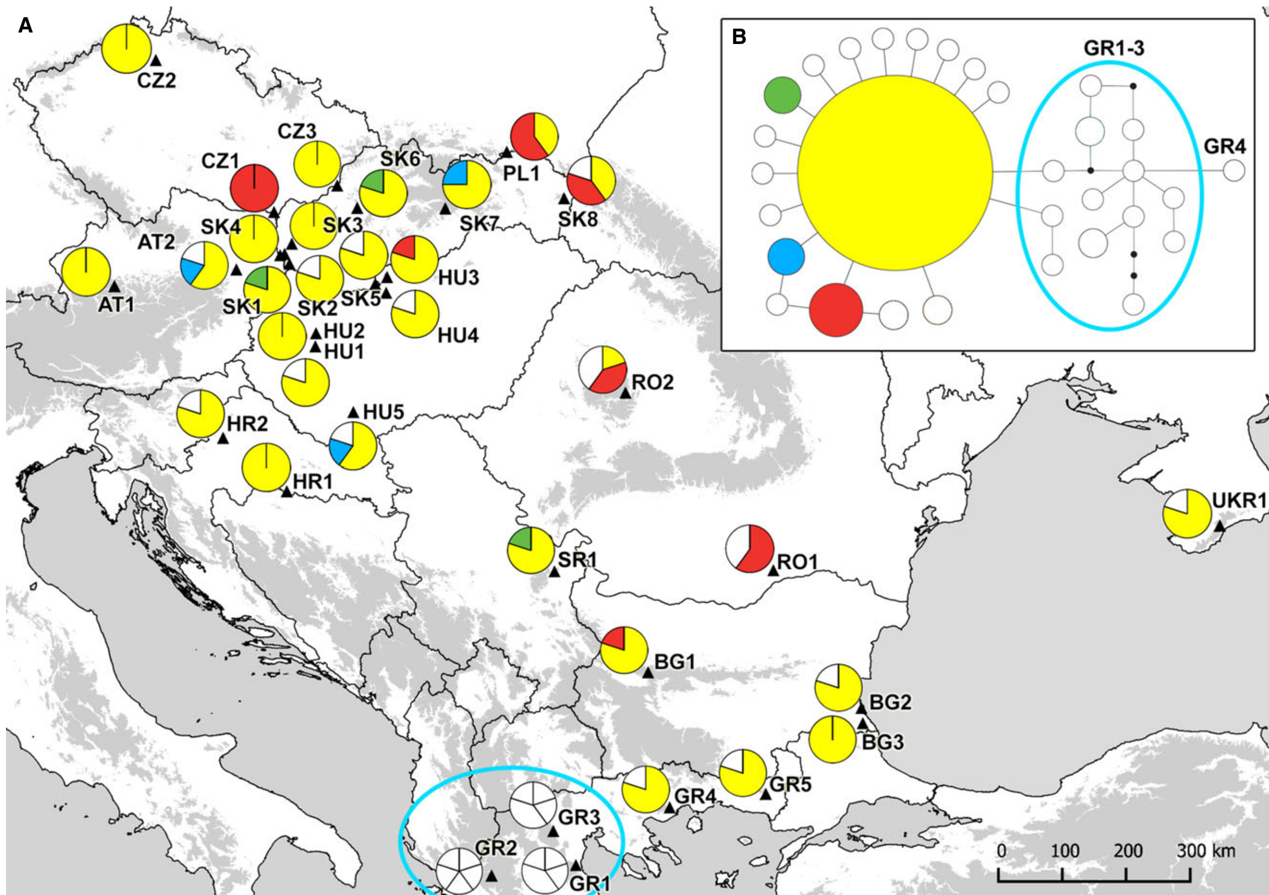

Figure 4. A, geographical distribution of the 31 haplotypes among the 33 sampled populations (full names are listed in Table 1) of Rosalia longicorn (*Rosalia alpina*) based on cytochrome *c* oxidase subunit I (COI) sequences. Each pie chart represents a proportion of haplotypes from a given population. Same haplotypes were marked by same colour (white parts represent haplotypes unique for a given population). Grey surface represents the area above 800 m a.s.l. B, haplotype network constructed using the statistical parsimony method (TCS); each haplotype is represented by a circle; the circle colour corresponds to the haplotype distribution map. The circle size is proportional to the haplotype frequency. Small black circles indicate missing haplotypes that are not present in the sample but are necessary to link all observed haplotypes to the network. Blue area highlights all haplotypes found only in three Greek populations (GR1, GR2, GR3).

existence of a glacial refugium of *R. alpina* in the high mountains of NW Greece (Pindos, Olymp). Our data do not enable us to determine whether the refugium was restricted only to the higher mountains of NW Greece, or whether it also reached the mountains along the eastern coast of the Adriatic Sea. The former scenario of the small glacial refugium is in line with the mountains of Greece acting as refugia for numerous other invertebrate species (Gratton, Konopinski & Sbordoni, 2008; Dinca *et al.*, 2013; Theissinger *et al.*, 2013). Nevertheless, the latter scenario of a larger refugium is supported by the high level of endemism on the eastern Adriatic coast and the presence of numerous glacial refugia there, including probably one of the beetle's main hosts: the European beech (*Fagus sylvatica*) (Magri *et al.*, 2006; Magri, 2008; Brus, 2010).

Based on the microsatellites, the cluster analysis indicated the existence of two genetically distinct lineages. The SE lineage consists of populations from Greece, Bulgaria, Romania, eastern Slovakia, and Poland. This lineage probably originates in the aforementioned glacial refugium. The NW lineage includes populations from central Austria, Czech Republic, western Slovakia, Hungary, Croatia, and, surprisingly, Ukraine (Crimea). The presence of the second lineage suggests the existence of another glacial refugium for this species. As has been shown for other organisms (Schmitt, 2009), there is no significant boundary between the Eastern Alps and the Western Carpathians. For *R. alpina*, it is rather unsurprising because the shortest distance between the studied populations from two montane systems (Wienerwald with population AT2 and Lesser

Carpathians with population SK3) is an approximately 50 km and the beetle also inhabits the lowland area between them. Our results suggest the two lineages met somewhere near the border between the Western and Eastern Carpathians. Such a pattern in which the Carpathians were probably colonized from several sources and acted as the contact zone has already been described for other insects (e.g. the meadow spittlebug *Philaenus spumarius*) (Lis *et al.*, 2014).

The mitochondrial (mt)DNA haplotype diversity also decreases with increasing latitude. This supports the assumption of a glacial refugium in NW Greece, thus corroborating the aforementioned results of nuclear DNA analyses. There are, however, some differences in the outcomes of the analyses of the two markers. The central mtDNA haplotype H1 was widespread and dominant in most of the study area. This may suggest that this area was colonized through the rapid expansion of a single lineage. The lineage might have originated in NW Greece, despite the H1 haplotype not being detected there. Given the high haplotype diversity in NW Greece, the rare presence of the H1 haplotype is likely in this region. It is interesting that all haplotypes found in NW Greece were restricted to this area; they probably did not contribute to the rest of the European gene pool, although the possibility of their spread along the Adriatic Sea cannot be ignored. Furthermore, the occurrence of the H3 haplotype was confined to the Carpathians and their vicinity. It is unclear whether the haplotype is a result of a single mutation in H1 that may have occurred during the colonization process, or whether it represents a separate lineage with its own refugium located in the Carpathians (as suggested for beech; Magri, 2008) or somewhere outside the study area. Interestingly, the transition between haplotypes H1 and H3 also occurs near the border between the Western and Eastern Carpathians, thus partly corroborating the results of the nuclear markers.

The distinctions between nuclear and mitochondrial markers are common and may be influenced by patterns of, for example, mating, sex-biased dispersal (Chesser & Baker, 1996; Miller, Haig & Wagner, 2005; Caparroz, Miyaki & Baker, 2009) or different evolutionary dynamics of the markers (Frankham *et al.*, 2002). Despite some discordance in results of both markers, the main patterns were similar. We may thus conclude that the populations in NW Greece deserve particular conservation efforts because they are the major genetic diversity reservoir of the species.

Low genetic structure of the NW lineage

The IBD was significant across all populations (UKR1 omitted), probably as a result of the higher genetic differentiation of the SE lineage. The genetic differentiation of populations from the NW lineage of *R. alpina* was, however, surprisingly low, despite that the analysis involved populations from sites as far as 620 km apart.

The genetically poorest population was the one inhabiting Crimean Peninsula (UKR1). Although its low AR might be partly resulting from the small sample size, it requires additional explanation. During the last glaciation, the Crimea was mostly covered by steppe vegetation (Atanassova, 2005) and the persistence of full forest cover over a significant area is rather unlikely (Cameron, Pokryszko & Horsák, 2013). This, together with the low AR and no private alleles in the *R. alpina* population, suggests the post-glacial recolonization of the Crimea by a limited number of immigrants. The second-least diverse population was found in the northern Czech Republic (CZ2). The population is confined to a very small area, having been isolated for decades from other known populations by hundreds of kilometres (Drag *et al.*, 2011). Although this population is currently relatively large, its low AR might be explained by the fluctuation in population size.

We failed to find any substructure within the NW lineage (NJ tree, cluster analysis). Despite all populations within the NW lineage being genetically rather poor, all of the microsatellite loci were polymorphic. The populations were nevertheless surprisingly uniform and displayed low incidence of private alleles. Thus, the lack of substructure within the lineage requires another explanation than that of the low genetic diversity. This suggests high population admixture over most of Central Europe and/or conservatism in genetic structure.

The high admixture suggests high gene flow of *R. alpina* populations within the NW lineage. It might be a result of the strong dispersal capacity of the species, the lack of migration barriers, and/or wood trade related accidental translocations. Genetic diversities of *R. alpina* do not resemble other saproxylic beetles with restricted dispersal ability such as flightless longhorn beetle *Morimus funereus* Mulsant, 1862 (Solano *et al.*, 2013) or saproxylic Hermit beetle *Osmoderma barnabita* Motschulsky, 1845 (Oleksa *et al.*, 2013). They are rather similar to highly mobile widespread saproxylic pests such as bark beetles (Cognato, Seybold & Sperling, 1999; Cognato, Harlin & Fisher, 2003; Avtzis, Arthofer & Stauffer, 2008; Horn *et al.*, 2009) or some rather mobile butterflies (Zakharov & Hellmann, 2008). Furthermore, considering the spatial scale investigated, the F_{ST} values based on microsatellites were rather low, typical for butterflies with high dispersal abilities (Williams, Brawn & Paige, 2003; Zakharov & Hellmann, 2008; Vandewoestijne & Van Dyck, 2010). Although the human-related translocations

might have contributed to this pattern, our findings are in agreement with the results of previous studies, suggesting that the *R. alpina* is an active, mobile species (Drag *et al.*, 2011). Moreover, the species' current distribution is continuous in many parts of the study area (e.g. Carpathians) and, together with its ability to also exploit lowland habitats (*see below*), this indicates a lack of migration barriers within the studied area during most of recent history.

With respect to the above, several populations with presumably restricted gene flow (the distance to the closest known population > 50 km) were analyzed (CZ2, UKR1, RO1, HU5). They were, however, genetically similar to other populations and lacked private alleles. This indicates a minimal effect of genetic drift typical for large populations (Frankham *et al.*, 2002). We hypothesize that the effective size of all presumably isolated populations was rather large during their existence, thus mitigating the effect of genetic drift. This would indicate that *R. alpina* is unable to exist in small, isolated populations, possibly as a result of some intrinsic reasons related to the species' biology or behaviour. Such constraints to population survival are common in other groups, including butterflies (Kadlec *et al.*, 2010), although their existence has not been described for beetles so far. This hypothesis would not only explain the surprisingly low genetic differentiation of *R. alpina* within Central Europe, but also might explain why a mobile species with the ability to exploit a broad range of habitats has disappeared from substantial part of its range (see above).

The above hypothesis, as well as the high mobility of the species inferred from genetic and mark–recapture (Drag *et al.*, 2011) data, imply that conservation measures applied on a large scale are likely to benefit the beetle's conservation more than local measures (Bosso *et al.*, 2013; Fahrig, 2013). More specifically, creating small patches of suitable habitats or corridors between habitat patches only several kilometres distant is less important than creating matrix of habitats consisting of larger patches of suitable habitat within the beetles reach, most likely up to 5–15 km. It is also important to note that management measures focusing on microhabitat creation on individual trees, such as pollarding and shredding (Russo, Cistrone & Garonna, 2011; Castro *et al.*, 2012; Sebek *et al.*, 2013), are likely to be more effective than the often recommended stand-focused measures such increase of rotation age or push for changes in tree species composition in large areas.

Uplands versus lowlands

Based on both microsatellites and mtDNA, no genetic differences were found between lowland and upland populations of *R. alpina*. Although the lowland populations from Western and South-eastern Europe were known for long (Picard, 1929; Serafim & Maincan, 2008), the species most likely appeared recently in Central European lowlands (Cizek *et al.*, 2009). Indeed, the low AR and presence of the single, otherwise rare mtDNA haplotype H3 in CZ1 (the population referred to by Cizek *et al.*, 2009) imply the founder effect and the population's recent establishment by a limited number of individuals, probably from the Carpathians. The recent establishment hypothesis is also supported by further spread of the population (Hovorka, 2011).

Two scenarios for colonization of Central European lowlands including colonization from nearby mountains and colonization by lowland population from the south were proposed by Cizek *et al.* (2009). Our results, based on the comparison of seven lowland and 26 upland populations, ruled out the colonization from the south and thus also militate against the existence of a separate lowland lineage. Most lowland populations in Central Europe were found within a few kilometres of the nearest upland population. This, together with the genetic composition, suggests independent colonization events from the nearby upland populations.

The beetle regularly exploits nonbeech hosts in the mountains (Michalcewicz & Ciach, 2012; Michalcewicz, Bodziarczyk & Ciach, 2013). This common ability to use nonbeech hosts most likely features in individuals of most, if not all, populations. Thus, no shift in host preference as suggested by Cizek *et al.* (2009) was needed to allow for the observed *R. alpina* expansion to lowlands. Although the climate change might have some effect (Müller *et al.*, 2015), it is more likely that the expansion of *R. alpina* to the lowlands has been facilitated by changes in the colonized habitat. *Cucujus cinnabarinus* (Scopoli, 1763) is another example of an endangered saproxylic beetle species that originally inhabited mid and higher altitudes, and has expanded to the lowlands of Central Europe in the last few decades (Horák, Chumanová & Hilszczański, 2012). Although the expansion of *C. cinnabarinus* has been attributed to an increase of the amount of poplar dead-wood, the expansion of *R. alpina* to lowlands might be attributed to increased availability of deadwood of shade tolerant trees such as ash (*Fraxinus* spp.) and maple (*Acer* spp.) (Marigo *et al.*, 2000; Garbarino *et al.*, 2014). Although the recent spread of the two species to lowlands of Central Europe is certainly beneficial for them, it may indicate changes in the tree species composition and spatial structure of lowland forests. Such changes have indeed been described as a transition from thermophilous, open, mainly oak

woodlands to closed canopy forests with more mesic conditions (Hédl, Kopecký & Komárek, 2010; Miklín & Čížek, 2014). Some saproxylic species certainly benefited from the change but this has led to a decrease in the habitat available for the substantially more diverse and endangered lowland fauna (Seibold *et al.*, 2014).

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Figure S1. Barplots gained from STRUCTURE ($K = 2$, $K = 3$, $K = 4$, $K = 5$, $K = 6$), and the number of clusters described as the highest number of ΔK (Evanno *et al.*, 2005) and the mean log probability of data LnP(D). We used 100 000 burn-in and 1 000 000 Markov chain Monte Carlo steps for 10 replicates for each K.
Table S1. Null allele frequencies for each locus and population estimated by FREENA.
Table S2. Pairwise F_{ST} values for 32 populatins created in ARLEQUIN with 10 000 permutations. The F_{ST} values with $P > 0.05$ are shown in red.