Postglacial distribution area expansion of *Polyommatus coridon* (Lepidoptera: Lycaenidae) from its Ponto-Mediterranean glacial refugium

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The genetic population structure of *Polyommatus coridon* (Poda, 1761) (Chalk-hill blue) was studied by means of allozyme electrophoresis in north-eastern Germany, the Czech Republic, Slovakia and Hungary. All analysed parameters showed high genetic diversity within populations (number of alleles: 2.61; observed and expected heterozygosity: 18.6% and 19.7%, respectively; percentage of polymorphic loci: total: 73.6%, on 95% level: 56.1%), whereas genetic differentiation between populations was comparatively low (F_{ST} = 0.028 ± 0.005 s.d.). Hierarchical variance analysis revealed significant structuring among five regional population clusters. A significant isolation-by-distance structure exists (r = 0.39; $P < 0.05$). The mean number of alleles per locus declined significantly from south to north and showed a strong correlation with the geographical latitude ($r = -0.88$, $P < 0.0001$). We suggest that this reflects the loss of alleles during the postglacial colonisation of eastern Central Europe from an ice-age refugium in the Balkans. A possible scenario for the postglacial expansion process in eastern Central Europe is discussed using these data: coming from the north-western part of the Balkans, *P. coridon* may have reached the western tip of Hungary, and consequently colonised eastern Central Europe using two alternative expansion routes.
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Introduction

Many thermophilic species were restricted to refugia in southern Europe during the last ice-ages (eg, Hewitt, 1996, 1999; Taberlet *et al*, 1998). In a previous work, we deduce the glacial isolation of *Polyommatus coridon* (Poda, 1761) (Chalk-hill blue) in two Mediterranean refugia: the Italian peninsula and the Balkans (Schmitt and Seitz, 2001a). This isolation apparently resulted in two well differentiated genetic lineages. For the western lineage, we previously analysed the postglacial expansion of the distribution area and pointed out possible corridors through which spread was possible into Western and western Central Europe (Schmitt *et al*, 2002).

For the eastern lineage, few data are available in the literature. None of the existing studies, dealing with allozyme analyses of *P. coridon*, included eastern populations (Mensi *et al*, 1988; Lelièvre, 1992; Marchi *et al*, 1996). The number of chromosomes was studied only in the Balkans (de Lesse, 1969).

The eastern lineage of *P. coridon* is distributed in the east as far as the steppes north of the Lake Caspi (Korschunov and Gorbunov, 1995). In the south, it reaches northern and central Greece (Pamperis, 1997), but is absent from Turkey (Hesselbarth *et al*, 1995). In Poland, *P. coridon* reaches the Baltic Sea (Buszko, 1997). In eastern Germany, the most northern populations are in north-eastern Brandenburg (Reinhardt, 1983). In the west, this lineage comes into contact with the western *P. coridon* lineage. Combining genetic and morphological studies, the boundary between both genetic lineages was found to be in eastern Thuringia. In the south, it follows the Czech-German and the Austrian-German border. In the Alps, the regions draining to the east are populated by the eastern lineage. The border between Slovenia and Italy represents the western limit south of the Alps (Schmitt and Seitz, 2001a). European distribution limits and the probable contact zone with the western lineage are shown in Figure 1.

This paper contributes to a better understanding of the postglacial distribution changes, the population structure and the genetic composition of the eastern lineage of *P. coridon*.

Materials and methods

A total of 743 butterflies was collected at 18 localities (see Figure 1), and stored in liquid nitrogen immediately after being captured until electrophoretic analysis was carried out. Furthermore, the density of the *P. coridon* population and the habitat size was assessed for each sampling site, and hereby, the total populations size was roughly estimated.

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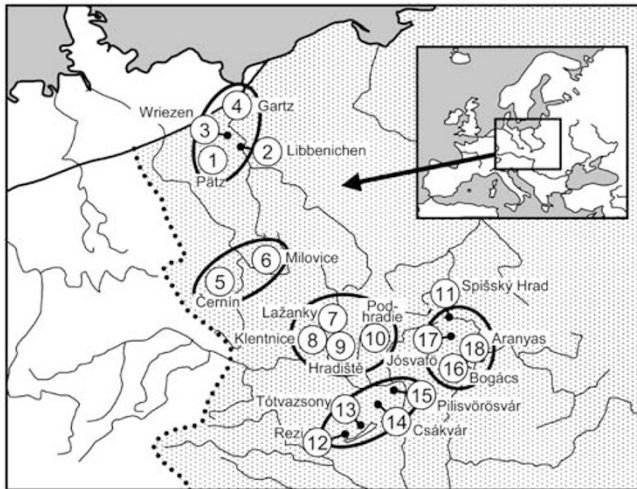
Figure 1 Sampling localities of *Polyommatus coridon*. Sample numbers correspond to Table 2. The species' northern distribution limit (Tolman and Lewington, 1998) is marked as a solid line, the probable border between the eastern and the western lineage conforming phenotypic differentiation (Schmitt and Seitz, 2001a) is shown as a dotted line, and the distribution range of the eastern lineage of *P. coridon* (Tolman and Lewington, 1998) is indicated by a dotted area.

Table 1 Density of the *Polyommatus coridon* populations studied, size of their habitat and estimated total number of individuals

		Density	Area (ha)	Total no. of individuals
Pätz	G-BB	m–h	5–10	200–1.000
Libbenichen	G-BB	m	~100	>1.000
Wriezen	G-BB	m	>100	>1.000
Gartz	G-BB	l	>100	~1.000
Černín	CZ-B	m–h	2–5	~1.000
Milovice	CZ-B	l–m	>100	>1.000
Lažanky	CZ-M	h	~2	200–1.000
Klentnice	CZ-M	l–m	~100	~1.000
Hradiště	SK-SW	h	~100	>10.000
Podhradie	SK-SW	l	~100	~1.000
Spišsky Hrad	SK-E	l–h	~50	>1.000
Rezi	H-W	m	~50	>1.000
Tótvazsony	H-W	m–h	>100	>10.000
Cszákvár	H-W	m–h	>100	>10.000
Pilisvörösvar	H-W	l	~100	~1.000
Bogács	H-NE	h	~10	>1.000
Jósvafö	H-NE	l	>100	>1.000
Aranyas	H-NE	l	>100	>1.000

The density was calculated using three density classes (l: low, ~10 individuals/ha; m: medium, ~50 individuals/ha; h: high, ≥100 individuals/ha, locally even reaching densities of 1.000 and more individuals per ha). For abbreviations of regions (column 2) see Figure 2.

Allozyme electrophoresis of twenty loci was performed using standard protocols (eg, Richardson *et al*, 1986; Hebert and Beaton, 1993). Details on enzymes and running conditions are described in Schmitt and Seitz (2001b). The discrimination between some alleles of Ldh was not always possible. Therefore, the results for this enzyme were excluded from all calculations of genetic distances and identities and from all further calculations based on these values.

All loci showed banding patterns consistent with known quaternary structures and with autosomal inheritance (Richardson *et al*, 1986) except for 6Pgdh which is located on the Z-chromosome (see Schmitt and Seitz, 2001a). The alleles were labelled conforming their relative mobility.

The allele frequencies, F-statistics (Weir and Cockerham, 1984), Nei's standard genetic distances (Nei, 1978) and RxC tests (Sokal and Rohlf, 1995) were calculated with G-Stat (Siegismund, 1993). Hierarchical variance analysis was done with Arlequin 2.000 (Schneider *et al*, 2000). Hardy-Weinberg equilibrium (Louis and Dempster, 1987), genetic disequilibrium (Weir, 1991), exact tests for differentiation (Raymond and Rousset, 1995a) and Mantel tests (1967) were performed with GENEPOP (Raymond and Rousset, 1995b). Spatial autocorrelation was analysed with Le Progiciel R 4.0d3 (Casgrain and Legendre, 2001). Un-rooted cluster analyses were done with PHYLIP (Felsenstein, 1993). We used the neighbour joining (Saitou and Nei, 1987) and the UPGMA method. Differences between means were tested by two-tailed U-tests and sign-tests using STATISTICA (Stat Soft, 1993).

Results

Population sizes
Most of the populations studied had largely similar numbers of individuals (see Table 1). Only two populations were particularly small (Pätz, Lažanky) and three populations particularly big (Hradiště, Tótvazsony, Csákvár). The population size of all the others was about 1000. Fourteen of the habitat areas were fairly large (ie, at least 50 ha). The remaining four habitats (Pätz, Černín, Lažanky, Bogács) were between two and 10 ha. Populations of high and low densities were randomly distributed over the whole study area.

Genetic variability
All 20 loci were polymorphic in at least two of the 18 populations. The maximum number of distinguishable alleles was 12 in Pgi; while in Apk, only two alleles were detected. The mean number of alleles per locus for all populations was 6.8 (± 2.9 s.d.). All allele frequencies are available on request from the authors.

The average number of alleles detected per locus per population showed a mean of 2.61 (± 0.34 s.d.), ranging from 1.9 to 3.2. The percentage of polymorphic loci ranged from 55% to 85% with a mean of 73.6% (± 8.7 s.d.). Applying the 95% criterion, the mean number of polymorphic loci was 56.1% (± 5.8 s.d.), and ranged from 45% to 65%. The high percentage of polymorphic loci coincided with high observed heterozygosities, ranging from 15.7% to 20.6%, with a mean of 18.6% (± 1.3 s.d.). Expected heterozygosity (19.7% (± 1.5 s.d.), ranging from 16.2% to 21.9%) was marginally but significantly higher (sign test: $P = 0.0095$). All data are presented in more detail in Table 2.

Hardy-Weinberg equilibrium and linkage disequilibrium
No significant deviation from Hardy-Weinberg equilibrium was observed for any of the populations or loci. No significant linkage disequilibrium was found after applying the Bonferroni correction. Therefore, further analyses were performed applying standard methods of population genetics.

Table 2 Average number of alleles per locus (A), expected (H_e) and observed heterozygosity (H_o), total polymorphic loci (P_{tot}) and on 95%-level (P_{95}) for all analysed samples of eastern European *Polyommatus coridon*

	1 G Pätz	2 G Libbe	3 G Wriez	4 G Gartz	5 CZ Černín	6 CZ Milov	7 CZ Lažan	8 CZ Klent	9 SK Hradiš	10 SK Podhr	11 SK Spišsk	12 H Rezi	13 H Tótvaz	14 H Czákv	15 H Pilis	16 H Bogác	17 H Jósva	18 H Arany
A	2.000	2.350	2.400	1.900	2.400	2.550	2.550	2.750	2.800	2.450	2.400	2.900	3.150	2.900	3.000	2.900	2.750	2.800
H_e	0.195	0.198	0.162	0.170	0.193	0.202	0.197	0.219	0.184	0.218	0.191	0.207	0.218	0.185	0.207	0.201	0.190	0.203
H_o	0.199	0.184	0.157	0.161	0.174	0.195	0.190	0.189	0.196	0.199	0.185	0.206	0.195	0.188	0.200	0.181	0.181	0.197
P_{tot}	0.550	0.800	0.750	0.550	0.700	0.700	0.800	0.750	0.800	0.800	0.650	0.750	0.850	0.750	0.800	0.800	0.800	0.650
P_{95}	0.550	0.650	0.450	0.500	0.550	0.650	0.500	0.650	0.600	0.600	0.500	0.600	0.550	0.550	0.550	0.550	0.500	0.600
n	48	40	40	33	45	50	40	47	45	36	50	40	40	40	39	40	31	39
capt	07	07	08	08	23	22	20	10	11	11	17	12	12	13	14	15	15	16/17

n = sample size; names of sampling localities are abbreviated. capt = day of capture in August 1997.

Regional structuring

Applying an exact test for differentiation, the allelic differentiation between samples was highly significant for all loci after Bonferroni correction ($P < 0.0001$). The genetic distances (Nei, 1978) among samples ranged from 0.015 (Hradiště, south-western Slovakia and Bogács, north-eastern Hungary) to 0.037 (Pätz, Brandenburg and Bogács, north-eastern Hungary). The mean genetic distance between populations was 0.022 ($\pm$ 0.003 s.d.). F_{ST} (0.028 $\pm$ 0.005 s.d.) and F_{IS} (0.037 $\pm$ 0.013 s.d.) differed significantly from zero. The total genetic variance was 1.883. Hierarchical analysis discriminating the five geographic population clusters (see Figure 1) revealed 22.4% of the among populations variance between these five groups.

The un-rooted cluster analyses, worked out with the neighbour-joining and the UPGMA method, revealed similar results. The populations from Hungary clustered predominantly together. The majority of populations from Czech Republic, Slovakia and Brandenburg formed a second cluster. The genetic distances of the Hungarian populations were greatest to the four populations from Brandenburg, especially to the population from Pätz. The neighbour-joining phenogram is shown in Figure 2.

Hierarchical variance analysis supported the topology of the neighbour joining tree: an analysis testing the western group (western Slovakia, Czech Republic, Brandenburg) against the eastern group (Hungary, eastern Slovakia) revealed 18.7% ($P = 0.019$) of the between populations variance between these two groups. Within both of these groups, the variance between their internal geographical clusters was not significantly different from zero (western: $P = 0.176$; eastern: $P = 0.404$).

A correlation analysis revealed a significant positive relation between geographic and genetic distances of the samples ($r = 0.39$; Mantel-test: $P < 0.05$; see Figure 3). The resulting r^2 of 0.15 indicates that 15% of the total genetic differentiation between populations is explained by their geographical distances. Spatial autocorrelation analysis supported isolation-by-distance: for two loci (6Pgdh, G6Pdh), Moran's I declined continuously with increasing geographical distance.

The mean number of alleles per locus declined from the southern samples (eg, western Hungary with 2.99 ($\pm$ 0.12 s.d.)) towards the northern ones (eg, Brandenburg with 2.16 ($\pm$ 0.25 s.d.)). We found a strong correlation between geographical latitude of the sample stations and the mean number of alleles per locus ($r = -0.88$, $P < 0.0001$; see Figure 4). For the other tested parameters of
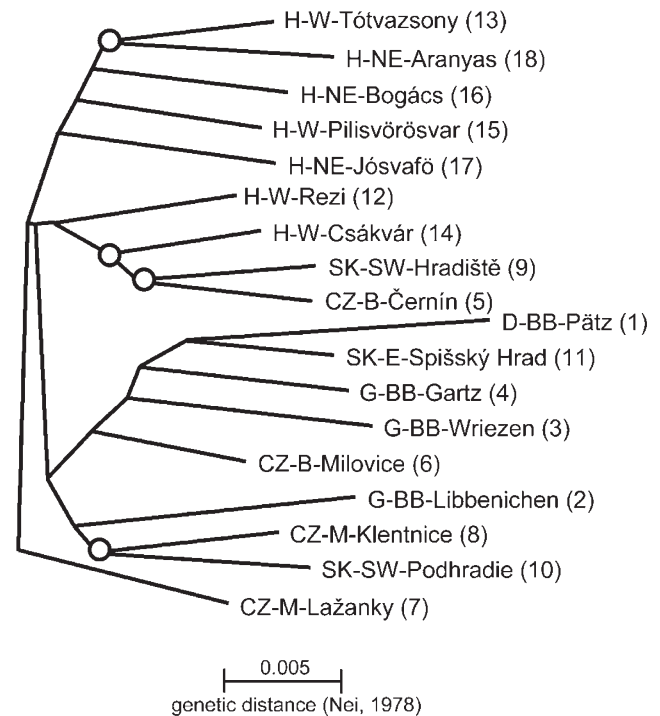

Figure 2 Neighbour-joining diagram of eastern European populations of *Polyommatus coridon* based on genetic distances (Nei, 1978). All nodes are significant on the 5% confidence level before Bonferroni correction. Those not significant after Bonferroni correction are marked with an open circle. The significance refers to RxC χ^2 tests of homogeneity of allele frequencies. Abbreviations: *First part*: country; *Second part*: BB: Brandenburg, B: Bohemia, E: East, NE: North-East, M: Moravia, SW: South-West, W: West; Third part: name of sampling locality. The population numbers in parenthesis refer to those in Figure 1.

genetic diversity, we obtained weaker correlation (observed heterozygosity: $r = -0.61$, $P = 0.007$; expected heterozygosity: $r = -0.59$, $P = 0.010$; total percentage of polymorphic loci: $r = -0.54$, $P = 0.020$; polymorphic loci at the 95% level: $r = -0.18$, $P = 0.466$). The total variance was also highest in the western Hungarian populations cluster and lowest in the Brandenburg populations cluster (see Table 3).

The four samples from western Hungary had a significantly higher mean number of alleles per locus (2.99 $\pm$ 0.12) than the four from north-eastern Hungary and eastern Slovakia (2.71 $\pm$ 0.22) (U-test: $P = 0.043$). The two
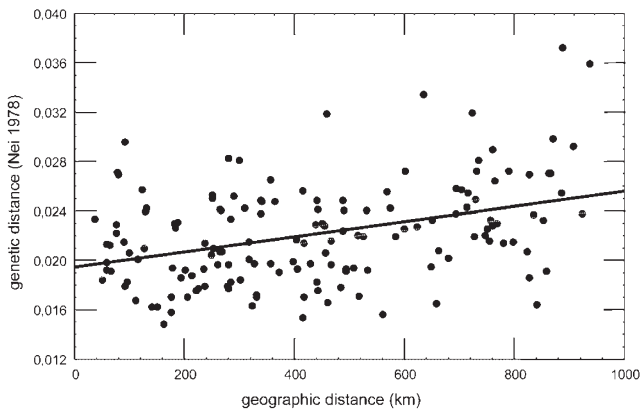
Figure 3 Relation between geographic and genetic distances of eastern European *Polyommatus coridon* populations (correlation analysis: $r = 0.39$; Mantel-test: $P < 0.05$).
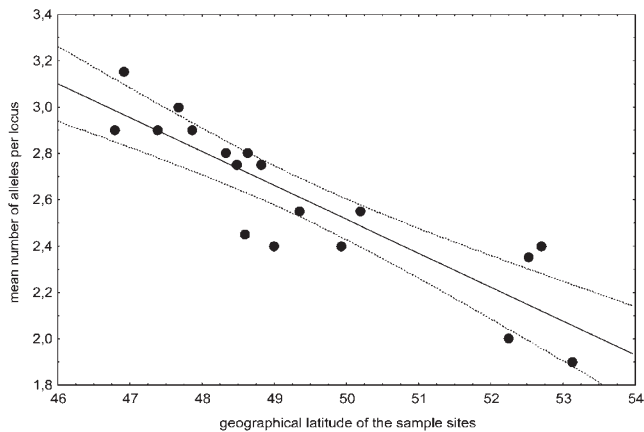

Figure 4 Correlation between geographical latitude of the sample site and the mean number of alleles of eastern European *Polyommatus coridon* ($r = -0.88$, $P < 0.0001$). The solid line shows the regression and the dotted lines the 95% confidence interval.

Table 3 Genetic variance within and between populations and F_{ST} values for the five regional population clusters of eastern European *Polyommatus coridon*

	Genetic variance			F_{ST}
	Total	*Between*	*Within*	
WH	1.931**	0.016*	1.915*	0.0083*
NEH/ESK	1.910**	0.045**	1.865**	0.0237**
Mora/WSK	1.919**	0.041**	1.878**	0.0213**
Bohemia	1.905**	0.027*	1.878**	0.0140*
Brandenburg	1.705**	0.080**	1.625**	0.0471**

Abbreviations: between = between populations; within = within populations; WH = western Hungary; NEH/ESK = north-eastern Hungary and eastern Slovakia; Mora/WSK = Moravia and western Slovakia. *$P < 0.005$; **$P > 0.0001$.

populations sampled in Bohemia showed a lower mean number of alleles (2.48 ± 0.11) than the four populations that originated from Moravia and western Slovakia (2.63 ± 0.17) (U-test: $P = 0.25$).

Discussion

Genetic variation within populations of *P. coridon*
In relation to other butterfly species, eastern European populations of *P. coridon* displayed a high level of genetic diversity. In other surveys on lepidoptera, in general, lower values were found. Thus, higher mean numbers of alleles were observed in only a very limited number of studies (eg, Sluss *et al*, 1978; Vandewoestijne *et al*, 1999). Also the other parameters reflecting genetic heterogeneity were higher than the average of other analysed butterfly species. For more detailed data see Graur (1985), Packer *et al* (1998) and Schmitt *et al* (2002).

Genetic differentiation among populations
The obtained F_{ST} (0.028 ± 0.005 s.d.) and the means of genetic distances (Nei, 1978) between the studied populations of *P. coridon* (0.022 ± 0.003 s.d.) are average values for lepidopterans (cf. Emelianov *et al*, 1995). These two values were similar to western European *P. coridon* (F_{ST}: 0.021, mean of genetic distance between populations: 0.020; Schmitt *et al*, 2002). Higher values for both parameters have been observed for species that include more than one subspecies, such as the Californian *Coenonympha tullia* assemblage (F_{ST}: 0.051, maximum genetic distance between genetic lineages: 0.034; Porter and Geiger, 1988), *Euphydryas editha* in the Great Basin and the Rocky Mountains (F_{ST}: 0.209, genetic distance between the two major lineages: 0.033; Britten *et al*, 1995) and the two host races of *Zeiraphera diniana* (F_{ST}: 0.083, maximum genetic distance between lineages: 0.04; Emelianov *et al*, 1995). In not subspecifically differentiated lepidopterans, the genetic distances were mostly smaller than in eastern populations of *P. coridon* (eg, Daly and Gregg, 1985; Rosenberg, 1989; Britten *et al*, 1994). F_{ST} values were higher in species showing a more patchy distribution than *P. coridon* (eg, *Speyeria nokomis apacheana* within regions: 0.227, Britten *et al*, 1994; *Euphydryas gilettii*: 0.375, Debinski, 1994; *Parnassius phoebus*: 0.225, Descimon, 1995; *Melitaea didyma*: 0.077, Johannesen *et al*, 1996). In migrating or widespread species, the F_{ST} values were lower than in our study or similar (eg, *Heliothis virescens*: 0.002, Korman *et al*, 1993; *Pieris napi napi*: 0.023, Porter and Geiger, 1995; *Helicoverpa armigera*: 0.007, Nibouche *et al*, 1998; *Aglais urticae*: 0.030, Vandewoestijne *et al*, 1999). Summing up, the eastern *P. coridon* lineage had a relatively weak genetic differentiation considering the extensive sampling area and the patchiness of habitats. The high population density in suitable habitats might prevent a stronger differentiation among populations due to the lack of genetic bottlenecks, and thus the conservation of once acquired genetic compositions.

Isolation-by-distance
The geographical distance between populations contributed significantly to the genetic differentiation between eastern populations of *P. coridon* ($r^2 = 0.15$). This is supported by the observed continuously decreasing spatial autocorrelation for two loci.

The degree of isolation-by-distance is similar to that found for *Chazara briseis* on local scale in Sachsen-Anhalt, eastern Germany (Johannesen *et al*, 1997). For seven populations of *Euphydryas editha gunnisonensis* in the Rocky Mountains, around 59% of genetic differentiation could be explained by isolation-by-distance, while 11

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Euphydryas editha-populations from the Great Basin showed no significant relationship (Britten *et al*, 1995). In *Pieris napi meridionalis*, even 64% of the differentiation could be explained by this phenomenon (Geiger and Shapiro, 1992). Thus, the observed structure gauged by isolation-by-distance of eastern populations of *P. coridon* is not very pronounced in comparison with other taxa (cf. Peterson and Denno, 1998).

Postglacial expansions

The number of alleles per locus declined constantly from western Hungary towards north-eastern Germany, correlating strongly with the geographical latitude. Seventy-eight percent of the decrease of alleles is explained by the geographical latitudes of the sample sites. We suggest that this is the result of a constant loss of alleles during the gradual and continuous postglacial colonisation of eastern Europe from an ice-age refugium in the Balkans. Most probably, this genetic erosion occurred mainly during the expansion because the number of colonisers in general is low while established populations of *P. coridon* generally have high population sizes.

A similar phenomenon was observed in the gallwasp *Andricus quercuscalicis* which expanded its distribution from south-eastern Europe to the British Isles during the last 400 years: the distance from the centre of dispersal explained 78.5% of the variance of the expected heterozygosity and 84.7% of the variance of the mean number of alleles (Stone and Sunnucks, 1993). Also other examples of genetic impoverishment during expansions are known (eg, Highton and Webster, 1976; Schwaegerle and Schaal, 1979; Johnson, 1988; Aßmann *et al*, 1994; Demesure *et al*, 1996; Dumolin-Lapègue *et al*, 1997), however, rates of genetic loss are mostly less continuous than in *P. coridon* and *A. quercuscalicis*.

While the highest numbers of alleles were found in western Hungary, this area might have been the first one of the studied regions to be reached by *P. coridon*. We suggest that western Hungary was colonised from the north-eastern edge of the glacial refugium via the Dinaric limestone mountains (see Figure 5, path 1). An immigration via the Iron Gap (passage of the Danube through the southern Carpathians (for their geographic location see Figure 5, 'IG') seems to be less probable: (1) The eastern part of the Carpathian Basin (especially the Transsylvanian region) offers unfavourable conditions for *P. coridon* because of soil conditions and other natural circumstances (Bálint, 1996). Nowadays, the species is scarce in that region, and also during the early Holocene a higher density of populations is unlikely due to the lack of extended limestone areas. Thus, rapid expansion through such a region seems unlikely. (2) On this route, (i) western Hungary and (ii) north-eastern Hungary/eastern Slovakia were the same expansion distance from the refugium. However, these two regions differ significantly in their mean number of alleles. (3) Furthermore, morphometric analyses of wing patterns show a high degree of homogeneity of populations from the north-western part of the Balkans to Hungary and further north to Brandenburg and Poland. Other Balkan populations show greater morphological heterogeneity (Schmitt, unpublished data).

The cluster analysis in the neighbour-joining phenogram revealed a differentiation into an eastern and a western group. This is supported by hierarchical variance
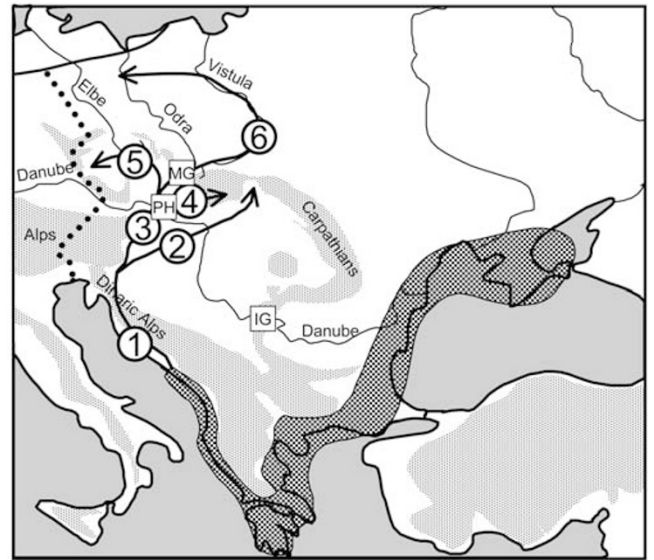

Figure 5 Postulated postglacial expansion routes of *Polyommatus coridon*. The assumed refugium is shown as a dark shaded field and assumed expansion corridors by arrows. Mountain regions are grey shaded. The dotted line represents the western border of the distribution range of the eastern genetic lineage. The solid line represents the northern distribution limit of the species. Abbreviations: IG: Iron Gap; PH: Porta Hungarica; MG: Moravian Gap; numbered expansion pathways are explained in the text.

analysis revealing significant deviation between these two groups. Within these groups, no hierarchical structure was detected. This suggests a split into two alternative expansion routes within eastern Europe (Figure 5): (1) from the western tip of Hungary following the Hungarian mountain chain to north-eastern Hungary and, later on, to eastern Slovakia (path 2) and (2) along the eastern Alps' foreland (path 3) and via the Porta Hungarica (for geographic location see Figure 5, 'PH') to western Slovakia (path 4) and the Czech Republic (path 5).

The populations from Brandenburg show strong effects of genetic erosion presumable having taken place during the expansion process: *P. coridon*, possibly, passed the Moravian Gap in north-eastern Moravia (for geographic location see Figure 5, 'MG') to southern Poland where it reached the Vistula. Here, primary expansion might have followed the Vistula river system. Brandenburg might have been colonised from Poland by a westward migration via the Thorn-Eberswald glacial valley (Figure 5, path 6). An alternative colonisation of Brandenburg could have taken place along the river Odra. We assume this possibility less probable because of less favourable natural conditions in this region (eg, soils are too acid for the larval food plants). Similar expansion corridors were also suggested for Ponto-Mediterranean dragonfly species (Sternberg, 1998).

Postglacial expansion effects might also explain why Bohemian populations are less diverse than Moravian and western Slovakian ones, since the second region is closer to the glacial refugium. Therefore, Bohemian populations should be derived from those from Moravia and western Slovakia. Loss of genetic diversity might have occurred during the north-westwards expansion. To clarify this question definitively, more samples from the Czech Republic and western Slovakia will have to be analysed.

Acknowledgements

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