INTRODUCTION

The climatic oscillations during the Pleistocene strongly influenced the distribution of most animal and plant species, and many thermophilic organisms were restricted to lower geographical latitudes during the cold glacial periods (Reinig, 1938; de Lattin, 1967; Hewitt, 2000). For Europe, the Mediterranean peninsulas were suggested to have been the most important refugial and differentiation centres for temperate species (de Lattin, 1967; Müller, 1980) as recently proved by numerous genetic analyses (Comes & Kadereit, 1998; Taberlet et al., 1998; Hewitt, 1999, 2000). However, these Mediterranean refugia did not represent homogeneous areas of glacial survival but were themselves strongly substructured, as

Furthermore, a remarkable part of the European fauna was considered in the past to be of eastern origin, e.g. as Siberian faunal elements (i.e. post-glacial immigration of the Western Palearctic from a glacial centre of survival in the Eastern Palearctic) (de Lattin, 1967; Müller, 1980). However, recent phylogeographical studies underline that many of these species survived the last ice age in Europe and that so-called extra-Mediterranean differentiation centres have been more important than previously thought (Hewitt, 2004). Therefore, these centres have not only been of major importance for the great majority of the mountain and arctic species (e.g. Després et al., 2002; Kropf et al., 2002, 2003; Stehlík, 2002; Schönswetter et al., 2003a,b,c, 2004; Ruokonen et al., 2005), but also for temperate species, as recently reported for the frog, *Rana arvalis* (Rafinkí & Babík, 2000; Babík et al., 2004), the fish, *Cottus gobio* (Engelbrecht et al., 2000), the voles, *Microtus agrestis* (Jaarola & Searle, 2002) and *Clethrionomys glareolus* (Deffontaine et al., 2005), the ant, *Formica pratensis* (Goropashnaya et al., 2004), the slug, *Arion fuscus* (Pinceel et al., 2005), the butterfly, *Erebia medusa* (Schmitt & Seitz, 2001a) or the nematode, *Heligmosomoides polygyrus* (Nieberding et al., 2005).

In the light of these results, the importance of south-eastern Europe as a centre of glacial survival and differentiation might be even greater than previously thought, because not only might the classical Pontic-Mediterranean refugial areas along the coasts of the Balkan Peninsula have served as centres of survival for various temperate species (cf. de Lattin, 1967; Müller, 1980), but also the continental parts of the Balkans and parts of the Carpathian region become candidates as important areas for glacial survival of a greater number of temperate species (e.g. Szymura et al., 2000; Schmitt & Seitz, 2001a; Babík et al., 2004; Goropashnaya et al., 2004; Deffontaine et al., 2005; Pinceel et al., 2005; Stoëv, in press).

However, comprehensive studies all over that area are rare, and therefore little is known about the detailed regional phylogeography of south-eastern Europe. A good model species to unravel such structures might be the woodland ringlet, *E. medusa* (Denis & Schiffermüller 1775). Biogeographers have considered this species as a ‘Siberian’ element (Osthelder, 1925; de Lattin, 1957; Varga, 1977), but intensive allozyme studies in Central Europe revealed a remarkable genetic structure of *E. medusa* giving strong evidence for several extra-Mediterranean glacial differentiation centres in Europe. At least three of these were located around the glaciated Alps (most probably west, south and south-east). A fourth lineage has been recorded in the form of populations originating from north-eastern Hungary, Slovakia and as far west as the western Czech Republic. The glacial differentiation centre of this lineage was previously supposed to have been located in south-eastern Europe (Schmitt & Seitz, 2001a).

In this paper we present an allozyme study of 615 *E. medusa* individuals from 28 populations scattered all over the species’ Romanian and Bulgarian distribution range to answer the following questions:

1. did *E. medusa* have one or more extra-Mediterranean differentiation centres in south-eastern Europe?
2. did the species survive the last ice age (1) north of the Danube in the Carpathian area and (2) south in the continental region of the Balkan peninsula?
3. has the Danube Valley been an important barrier for gene flow over a long time period?
4. did *E. medusa* have multiple differentiation centres of rather limited geographical extension in Bulgaria and Romania?
5. are different climatic conditions in these centres of survival reflected in different population genetic parameters?
6. might the woodland ringlet have survived the last ice age even within the Carpathian Basin?

The study species *Erebia medusa*

The woodland ringlet, *E. medusa* (Satyrinae, Nymphalidae, Lepidoptera) is a temperate butterfly species distributed from western Europe throughout temperate, Asia reaching the Pacific Ocean as its eastern distribution limit (Korschunov & Gorbunov, 1995; Tolman & Lewington, 1997; Kudrná, 2002). In Europe, the species avoids the eumediterranean, the euatlantic and the northern boreal regions (Henriksen & Kreutzer, 1982; Emmet & Heath, 1990; Fernández-Rubio, 1991; Bink, 1992; Kudrná, 2002), but is widely distributed in south-eastern Europe (Jaksic, 1988; Pamperis, 1997; Rákosy, 1998; Abadjiev, 2001; Kudrná, 2002; Rákosy et al., 2003). The larvae and imagos are found in a great variety of habitats including meadows, pastures and forest clearings (SBN, 1987; Ebert & Rennwald, 1991; Tolman & Lewington, 1997), in south-eastern Europe predominantly in mountainous areas (Rákosy, 1998; Abadjiev, 2001). However, all suitable habitats have in common: (1) the absence of major disturbance during and shortly after the flight season (e.g. mowing, intensive grazing), and (2) soils poor in nutrients (e.g. low amounts of available nitrogen, phosphate and potassium) (Schmitt, 2002).

**MATERIALS AND METHODS**

We analysed a total of 615 individuals of *E. medusa* from 28 populations from Romania (259 individuals from 17 populations) and Bulgaria (356 individuals from 11 populations) (Fig. 1). The individuals were sampled in meadows and pastures from early June to late July of the year 2004. The butterflies were netted in the field, frozen alive in liquid nitrogen and stored under these conditions until analysis.

We used cellulose acetate plates for allozyme electrophoresis applying standard protocols (Richardson et al., 1986; Hebert & Beaton, 1993). A total of 19 allozyme loci was analysed (for loci
studied and electrophoretic conditions see Schmitt & Seitz, 2001a). Most loci were autosomal, but 6Pgdh, Mpi and Me2 are located on the Z chromosome (Schmitt & Seitz, 2001a; T. Schmitt, unpublished data); thus, hemizygous females only have a single copy. However, the percentage of analysed females was low (mean 9.3%). Therefore, Z-chromosome linkage did not interfere with further analysis among populations.

We analysed the obtained allozyme data with three programmes: (1) G-STAT (Siegismund, 1993) for allele frequencies, parameters of genetic diversity of the populations (i.e. mean number of alleles per locus and population \( A \), expected and observed heterozygosity \( H_e \) and \( H_o \), respectively), total percentage of polymorphic loci \( P_{\text{tot}} \) and percentage of polymorphic loci with the most common allele not exceeding 95% \( P_{95} \) and genetic distances (Nei, 1978), (2) ARLEQUIN 2.000 (Schneider et al., 2000) for hierarchical variance analysis and \( F \) statistics, tests on differentiation, Hardy–Weinberg equilibrium and linkage disequilibrium, and (3) PHYLIP (Felsenstein, 2000) for the construction of trees based on genetic distances. Bootstraps based on 1000 iterations were calculated with the same software. The parameters of genetic diversity of the populations were only included into statistics if a minimum number of individuals was analysed (i.e. 25 individuals for \( A \), 10 individuals for \( P_{\text{tot}} \) and \( P_{95} \), 5 individuals for \( H_e \) and \( H_o \)). Standard statistics (\( U \)-tests, Kruskal–Wallis ANOVAS) were calculated using STATISTICA.

RESULTS

Seventeen of the 19 analysed loci were polymorphic and had banding patterns consistent with known quaternary structures. Only two loci (Me1 and Fum) were monomorphic throughout. We calculated the following results for the means of five population genetic parameters: mean number of alleles per locus \( A \), 2.05; expected heterozygosity \( H_e \), 15.6%; observed heterozygosity \( H_o \), 14.5%; total percentage of polymorphic loci \( P_{\text{tot}} \), 55.6%; percentage of polymorphic loci with the most common allele not exceeding 95% \( P_{95} \), 41.4% (see Table 1 for detailed data).

No deviation from Hardy–Weinberg expectations was calculated for any of the autosomal loci (all \( P > 0.05 \) after Bonferroni correction). No general linkage disequilibrium was observed between any pair of loci. Therefore, we performed further analyses using standard algorithms in population genetics.

The total genetic variance was 1.812, of which 1.564 (i.e. 86.3%) was within populations and as much as 0.248 among populations (i.e. an overall \( F_{\text{ST}} \) value of 13.7%, \( P < 0.0001 \)). Similar results were obtained excluding all samples of fewer than 14 individuals (genetic variance 1.814; overall \( F_{\text{ST}} \) 12.6%, \( P < 0.0001 \)). \( F_{IS} \) was 7.81% (only populations with > 14 individuals 7.42%; both \( P < 0.0001 \)) calculated for all loci, but dropped to 1.41% excluding the Z-chromosomal loci 6Pgdh, Mpi and Me2, and none of the single-locus \( F_{IS} \) values of the autosomal loci were significant (all \( P > 0.05 \)).

Based on genetic distances (Nei, 1978), a neighbour-joining analysis (only populations of seven or more individuals included) (Fig. 2) revealed a strong differentiation among populations. The split between the Romanian and the Bulgarian samples is supported by the highest bootstrap value (93.1), and the mean genetic distance (Nei, 1978) between these two population groups is 0.074 (±0.029 SD). This is reflected in the
distribution of variance: about 52.1% (only populations ≥ 14 individuals 54.7%) of the total variance among populations is distributed between these two countries ($F_{CT}$ 9.13%; $F_{SC}$ 9.22%; only populations ≥ 14 individuals, $F_{SC}$ 8.98%, $F_{SC}$ 8.16%; all $P < 0.0001$). This difference is well reflected in the allele frequencies of several loci (e.g. $Idh1$, $Mdh1$, $Mdh2$, $G6pdh$

<p>| Parameters of genetic diversity of the 19 populations (with five or more individuals) of Erebia medusa from Romania and Bulgaria. Values in parentheses are not included in means. Means are given with their standard deviations. |
|-----------------------------------|----------|----------|----------|----------|----------|----------|</p>
<table>
<thead>
<tr>
<th>Population</th>
<th>$A$</th>
<th>$H_e$ (%)</th>
<th>$H_o$ (%)</th>
<th>$P_{95}$ (%)</th>
<th>$P_{tot}$ (%)</th>
<th>$n$</th>
<th>Date of capture</th>
</tr>
</thead>
<tbody>
<tr>
<td>RO-WK Valea Draganului</td>
<td>1.68</td>
<td>18.5</td>
<td>17.7</td>
<td>42.1</td>
<td>47.4</td>
<td>40</td>
<td>4 June 2004</td>
</tr>
<tr>
<td>RO-EK-NW Bistrita Bargaului</td>
<td>1.79</td>
<td>19.7</td>
<td>19.1</td>
<td>42.1</td>
<td>47.4</td>
<td>40</td>
<td>6 June 2004</td>
</tr>
<tr>
<td>RO-EK-NE Vatra Moldovitei</td>
<td>1.68</td>
<td>20.5</td>
<td>18.0</td>
<td>47.4</td>
<td>47.4</td>
<td>40</td>
<td>7 June 2004</td>
</tr>
<tr>
<td>RO-EK-Rodna Valea Vinului</td>
<td>1.79</td>
<td>20.2</td>
<td>16.8</td>
<td>47.4</td>
<td>47.4</td>
<td>40</td>
<td>26 July 2004</td>
</tr>
<tr>
<td>RO-SEK-Pass Oitutz</td>
<td>(1.53)</td>
<td>16.1</td>
<td>14.7</td>
<td>(42.1)</td>
<td>(42.1)</td>
<td>5</td>
<td>8 June 2004</td>
</tr>
<tr>
<td>RO-SEK Lacu Sacele</td>
<td>1.68</td>
<td>12.6</td>
<td>11.2</td>
<td>36.8</td>
<td>42.1</td>
<td>40</td>
<td>9 June 2004</td>
</tr>
<tr>
<td>RO-SEK Pass Bratocrea</td>
<td>(1.32)</td>
<td>12.1</td>
<td>12.8</td>
<td>(26.3)</td>
<td>(26.3)</td>
<td>7</td>
<td>10 June 2004</td>
</tr>
<tr>
<td>RO-SK-W Pass Apa Seaca</td>
<td>(1.47)</td>
<td>10.8</td>
<td>8.1</td>
<td>21.1</td>
<td>36.8</td>
<td>19</td>
<td>18 July 2004</td>
</tr>
<tr>
<td>RO-SK-Retazed Gura Zlata</td>
<td>(1.37)</td>
<td>7.4</td>
<td>6.4</td>
<td>26.3</td>
<td>31.6</td>
<td>14</td>
<td>25 June 2004</td>
</tr>
<tr>
<td>BG-SP-W Petrohan Pass</td>
<td>2.63</td>
<td>18.7</td>
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<td>40</td>
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</tr>
<tr>
<td>BG-SP-W Monasteristhete</td>
<td>2.11</td>
<td>16.2</td>
<td>13.7</td>
<td>42.1</td>
<td>63.2</td>
<td>40</td>
<td>19 June 2004</td>
</tr>
<tr>
<td>BG-SP-C Troyan Pass</td>
<td>2.58</td>
<td>16.2</td>
<td>14.7</td>
<td>47.4</td>
<td>78.9</td>
<td>40</td>
<td>18 June 2004</td>
</tr>
<tr>
<td>BG-Lozen German</td>
<td>1.95</td>
<td>14.7</td>
<td>12.7</td>
<td>42.1</td>
<td>52.6</td>
<td>36</td>
<td>13 June 2004</td>
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<tr>
<td>BG-OSogovo Igilika</td>
<td>2.21</td>
<td>16.8</td>
<td>16.8</td>
<td>36.8</td>
<td>73.7</td>
<td>40</td>
<td>14 June 2004</td>
</tr>
<tr>
<td>BG-Komjavo Batikal</td>
<td>(1.90)</td>
<td>16.6</td>
<td>13.0</td>
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<td>63.2</td>
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<td>14 June 2004</td>
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<td>2.11</td>
<td>14.9</td>
<td>15.5</td>
<td>36.8</td>
<td>47.4</td>
<td>40</td>
<td>15 June 2004</td>
</tr>
<tr>
<td>BG-Rila-W Rila Monastery</td>
<td>(1.47)</td>
<td>11.7</td>
<td>11.1</td>
<td>(36.8)</td>
<td>(36.8)</td>
<td>9</td>
<td>15 June 2004</td>
</tr>
<tr>
<td>BG-Pirin-N Predela Pass</td>
<td>2.21</td>
<td>17.2</td>
<td>18.9</td>
<td>36.8</td>
<td>63.2</td>
<td>40</td>
<td>16 June 2004</td>
</tr>
<tr>
<td>BG-Pirin-S Popov Livadi</td>
<td>(1.58)</td>
<td>15.3</td>
<td>14.8</td>
<td>(36.8)</td>
<td>(36.8)</td>
<td>8</td>
<td>16 June 2004</td>
</tr>
<tr>
<td>BG-Rhodopi-S Trigrad</td>
<td>2.21</td>
<td>16.5</td>
<td>14.9</td>
<td>57.9</td>
<td>73.7</td>
<td>40</td>
<td>17 June 2004</td>
</tr>
<tr>
<td>Mean</td>
<td>2.05 ± 0.32</td>
<td>15.6 ± 3.4</td>
<td>14.5 ± 3.5</td>
<td>41.4 ± 9.2</td>
<td>55.6 ± 14.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean RO</td>
<td>1.73 ± 0.06</td>
<td>15.3 ± 4.7</td>
<td>13.9 ± 4.6</td>
<td>37.6 ± 10.3</td>
<td>42.9 ± 6.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean BG</td>
<td>2.25 ± 0.23</td>
<td>15.9 ± 1.8</td>
<td>15.0 ± 2.4</td>
<td>44.4 ± 7.5</td>
<td>65.5 ± 10.6</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$A$, mean number of alleles per locus; $H_e$, expected heterozygosity; $H_o$, observed heterozygosity; $P_{95}$, percentage of polymorphic loci with the most common allele not exceeding 95%; $P_{tot}$, percentage of polymorphic loci; $n$, number of individuals analysed; RO, Romania (WK, western Carpathians; EK NW, eastern Carpathians, north-west; EK NE, eastern Carpathians, north-east; EK Roda, eastern Carpathians, Rodna Mountains; SEK, south-eastern Carpathians; SK W, southern Carpathians, west; SK Ret, southern Carpathians, Retazed Mountains); BG, Bulgaria (SP W, Stara Planina, west; SP C, Stara Planina, central; Lozen, Mount Lozen; Osogovo, Osogovo Mountains; Konyavo, Mount Konyavo; Rila N, Rila Mountains, north; Rila W, Rila Mountains, west; Pirin N, Pirin Mountains, north; Pirin S, Pirin Mountains, south; Rhodopi S, Rhodopi Mountains, south).

Figure 2 Neighbour-joining diagram based on genetic distances (Nei, 1978) calculated for all 19 analysed loci of the Erebia medusa populations sampled in Romania and Bulgaria. Numbers in parentheses indicate the numbers of individuals analysed. If no number is given, 40 individuals were analysed. Values at the nodes of the branches indicate bootstrap percentages from 1000 iterations. Only values above 50% are given. Only populations with seven or more analysed individuals are included in the cluster analysis.
Furthermore, the populations from Bulgaria showed significantly higher mean values for the mean number of alleles per population and the total percentage of polymorphic loci than the populations from Romania (all $P < 0.05$; Fig. 4), but no significant difference was calculated for the heterozygosity and the percentage of polymorphic loci at the 95% level.

The mean genetic distance among the Romanian samples (0.061 ± 0.026 SD) was considerably higher than among the Bulgarian samples (0.039 ± 0.017 SD). The overall $F_{ST}$ value was also considerably higher among the Romanian samples (13.6%, $P < 0.0001$; only populations ≥ 14 individuals 11.6%; both $P < 0.0001$) than among the Bulgarian ones (5.68%; only populations ≥ 23 individuals, 5.39%; both $P < 0.0001$). Thus,

**Figure 3** Geographical distribution of the allele frequencies in the locus $G6pdh$ of the 28 Erebia medusa populations in Romania and Bulgaria. Black, allele 2; hatched, allele 4; white, allele 6; grey, other alleles. The size of the circles corresponds to the number of individuals analysed (100%, 36–40 individuals; 80%, 14–23 individuals; 60%, 5–9 individuals; 40%, 1–3 individuals). Mountain regions with mountains higher than 1000 m a.s.l. are shaded.

**Figure 4** Geographical distribution of the percentage of polymorphic loci ($P_{pol}$) of the 16 Erebia medusa populations (≥ 10 individuals) in Romania and Bulgaria. Mountain regions with mountains higher than 1000 m a.s.l. are shaded.
the differentiation among the Romanian population groups is nearly as enhanced as between Romania and Bulgaria.

The strong genetic differentiation in Romania is reflected in four well-distinguished subgroups (see Fig. 2), which show high genetic distances among each other: (1) the Retezat Mountains (i.e. the westernmost part of the southern Carpathians; sample, Gura Zlata), (2) the western part of the southern Carpathians (sample Pass Apa Seaka), (3) the southeastern Carpathians (samples Lacu Sacele, Pass Bratocea), and (4) the western Carpathians and the northern part of the eastern Carpathians (samples Valea Draganului, Vatra Moldovitei, Bistrita Barganului, Valea Vinului). (Note that the branch connecting Pass Bratocea to the tree is also relatively long. However, this seems to be an artefact resulting from the relatively small sample size. In such cases, the branching point is of major interest. This branching point is quite near the sample Lacu Sacele, thus underlining the close relationship between these two samples.)

Hierarchical variance analysis revealed that 83.0% (only populations > 14 individuals 88.7%) of the variance among populations was among these four lineages. Therefore, $F_{CT}$ (15.4%, $P < 0.0001$) was much higher than $F_{SC}$ (3.72%, $P < 0.0001$) (only populations > 14 individuals, $F_{CT}$ 15.4%, $P < 0.0001$; $F_{SC}$ 2.31%, $P < 0.0001$). Furthermore, the mean genetic distance was considerably higher among these groups (mean 0.073 ± 0.016) than within them (mean 0.025 ± 0.011). This strong differentiation into these four groups is well reflected in several loci [Idh2, Mdh2, 6Pgdh, G6pdh (Fig. 3), Mpi (Fig. 5), Pgm, Pep, Aat1, Aat2, Pk]. The populations of the western Carpathians and the northern eastern Carpathians group had significantly higher genetic diversities than the populations from the other groups ($U$-tests: $H_o, P = 0.014; H_o, P = 0.014; P_{95}, P = 0.031; P_{95}, P = 0.019$; Fig. 4).

The differentiation in Bulgaria, although not as strong as in Romania, also showed a hierarchical structure. The neighbour-joining phenogram (Fig. 2) distinguished four groups: (1) the two samples from the western Stara Planina (samples Monastirshte, Petrohan Pass), (2) the samples from the Sofia region in the north (Mount Lozen) to the saddle between the Rila and the Pirin Mountains in the south (samples Predela Pass, Borovets, Iglika, German, Rila Monastery; note that Baikal geographically belongs to this group but is clustered separately in the phenogram), (3) the southern Pirin and southern Rhodopi Mountains (samples Trigrad, Popovi Livadi), and (4) the central Stara Planina (sample Troyan Pass) (Fig. 2). This structure is supported by the respective bootstrap values (ranging from 60 to 69%) and a hierarchical variance analysis with 59.2% of the genetic variance among populations being among these four groups ($F_{CT}$ 4.00%, $P = 0.002; F_{SC}$ 2.87%, $P < 0.0001$; only populations > 23 individuals, $F_{CT}$ 3.86%, $P = 0.002; F_{SC}$ 2.94%, $P < 0.0001$). Furthermore, the mean genetic distance was higher among these groups (mean 0.041 ± 0.018) than within them (mean 0.032 ± 0.012). As the differentiation in Bulgaria is lower than in Romania, the differences in the allele frequencies are less obvious, but some patterns can be seen in Mpi (Fig. 5), Pep and Pk.

The comparison of our samples from Romania and Bulgaria with previously published allozyme data for E. medusa populations from France, Germany, northern Italy, Czech Republic, Slovakia and Hungary (Schmitt & Seitz, 2001a) revealed a strong genetic differentiation between all pairs of population groups (Table 2). Furthermore, the differentiation between the western Carpathian–northern eastern Carpathian

Figure 5 Geographical distribution of the allele frequencies in the locus Mpi of the 28 Erebia medusa populations in Romania and Bulgaria. White, allele 2; black, allele 4; grey, other alleles. The size of the circles corresponds to the number of individuals analysed (100%, 36–40 individuals; 80%, 14–23 individuals; 60%, 5–9 individuals; 40%, 1–3 individuals). Mountain regions with mountains higher than 1000 m a.s.l. are shaded.
group and the Slovakian–north-eastern Hungarian group of the eastern lineage was strong (genetic variance among groups 0.1946, $F_{CT}$ 9.6%, $P < 0.0001$; genetic variance within groups 0.0775, $F_{CT}$ 4.2%, $P < 0.0001$).

### DISCUSSION

#### Genetic diversity and differentiation

The analysed parameters of genetic diversity of the *E. medusa* populations from south-eastern Europe had an intermediate position compared with other representatives of the subfamily Satyrinae (Douwes & Stille, 1988; Porter & Geiger, 1988; Porter & Shapiro, 1989; Goulson, 1993; Porter et al., 1995; Johannesen et al., 1997; Habel et al., 2005; Schmitt et al., 2005a) and butterflies in general (Napolitano et al., 1990; Debinski, 1994; Napolitano & Descimon, 1994; Britten et al., 1995; Descimon, 1995; Pelz, 1995; Peterson, 1995; Porter & Geiger, 1995; Johannesen et al., 1996; Brookes et al., 1997; Gadeberg & Boomsma, 1997; Meglécz et al., 1997; Jiggins & Davies, 1998; Packer et al., 1998; Schmitt & Seitz, 2001b; Schmitt et al., 2003).

However, this general assumption is somewhat misleading because the mean number of alleles and the percentage of polymorphic loci $P_{st}$ are both much higher in Bulgaria than in Romania. Thus, the mean genetic diversity of the populations in Romania is not higher than observed for populations from Central Europe (Schmitt & Seitz, 2001a) and is therefore rather below the mean genetic diversity of butterflies. In contrast, the values obtained in the Bulgarian populations even get close to those observed in some very diverse species like many Lycaenidae (Peterson, 1995; Brookes et al., 1997; Schmitt & Seitz, 2001b; Schmitt et al., 2003, 2005b), or the rather common and widespread Satyrinae species, *Maniola jurtina* (Schmitt et al., 2005a). This difference might originate in the biogeographical structure and hence the ancient distribution patterns of the species as discussed below.

The values yielded from $F$ statistics and the genetic distances (Nei, 1978) among the south-eastern European lineages of *E. medusa* are as high as obtained for populations from Central Europe (Schmitt & Seitz, 2001a). Hence, they are as high as or even higher than in other butterfly species with a pronounced intraspecific structure (Porter & Geiger, 1988; Napolitano & Descimon, 1994; Descimon, 1995; Schmitt & Seitz, 2001b; Schmitt et al., 2005a) or even sibling species (Porter et al., 1995; Schmitt et al., 2005b). If the velocities of differentiation in butterflies in general are of the same order of magnitude, the observed genetic differentiation within *E. medusa* in south-eastern Europe is not a post-glacial phenomenon, but was most likely initiated during or even prior to the last ice age.

### Molecular biogeography

The strong differentiation between Romania and Bulgaria makes a long-term separation without gene flow between these two regions the most likely scenario. Compared to the differentiation among Central European genetic lineages of *E. medusa* (Schmitt & Seitz, 2001a) as well as within other butterfly species (Porter & Geiger, 1988, 1995; Descimon, 1995; Meglécz et al., 1997; Schmitt & Seitz, 2001b; Schmitt et al., 2003, 2005a,b), the observed genetic diversification in south-eastern Europe strongly supports the proposition that the Danube Valley has acted as a barrier for the expansion of the woodland ringlet at least since the end of the Eem interglacial (about 130,000 yr bp). This would imply that this humid river valley did not offer suitable habitat conditions for *E. medusa*, during subsequent glacial and interglacial climatic conditions.

As the Bulgarian as well as the Romanian populations are well differentiated from all other known lineages (cf. Schmitt & Seitz, 2001a), the geographical location of the glacial differentiation centres should have some concordance with the actual genetic pattern. However, it is likely that a downslope translocation and thereby a restriction to the climatically more suitable lower hills and valleys has taken place during the last ice age (cf. Hewitt, 1996). Consequently, survival of *E. medusa* in Bulgaria as well as in the Carpathian area during the last ice age is the most likely scenario, hereby once more rejecting the old postulates of post-glacial expansion to Europe from a Siberian refugium (Osthelder, 1925; de Lattin, 1957; Varga, 1977).

Furthermore, the strong genetic differentiation of the populations originating from the Carpathian area and Bulgaria from the populations analysed from Czech Republic, Slovakia and north-eastern Hungary (the latter reported in Schmitt & Seitz, 2001a), rejects the previously postulated post-glacial Balkan origin of the latter group. Rather, further glacial differentiation centres have to be postulated for this group in southern Moravia and south of the northern Carpathians.

The significantly higher values for the mean number of alleles and the total percentage of polymorphic loci (i.e. the two parameters most affected by genetic impoverishment) in Bulgaria let us argue that the general conditions in this part of the Balkans have been more favourable than further north. The conditions in Romania might have been quite at the limit of the climatic tolerance for this species. Therefore, *E. medusa* has gone through bottlenecks in Romania during the last ice age, hence suffering remarkable genetic impoverishment. The

<table>
<thead>
<tr>
<th>FG</th>
<th>W</th>
<th>E</th>
<th>SA</th>
<th>WP</th>
</tr>
</thead>
<tbody>
<tr>
<td>RO</td>
<td>9.13%</td>
<td>11.4%</td>
<td>10.6%</td>
<td>25.0%</td>
</tr>
<tr>
<td>BG</td>
<td>–</td>
<td>12.5%</td>
<td>8.2%</td>
<td>32.0%</td>
</tr>
</tbody>
</table>

![Table 2](image-url)
general conditions in Bulgaria must have been more favourable, so that fewer or no population bottlenecks occurred and consequently more genetic diversity (especially evident in the existence of many rare alleles) has been preserved. However, survival in the Carpathian region through the Würm ice age has also been demonstrated for other temperate species (e.g. Szymura et al., 2000; Babik et al., 2004; Goropashnaya et al., 2004; Defontaine et al., 2005; Pinceel et al., 2005).

Higher genetic diversity in large compared with small populations is a commonly observed phenomenon (e.g. Billington, 1991; Buza et al., 2000; Hudson et al., 2000; Jäggi et al., 2000; Madsen et al., 2000; Schmitt & Seitz, 2002), but only rather few cases of different glacial survival conditions leading to different genetic diversities of intraspecific lineages are known (cf. Schmitt & Seitz, 2001a; Nieberding et al., 2005).

The strong differentiation among the Romanian lineages is also an indicator of a long-term genetic isolation of these groups, most probably since at least the onset of the last ice age (cf. Schmitt & Seitz, 2001a). In general, the topology of the neighbour-joining diagram is reflected in the geographical position of the populations (Fig. 6). Three of the four groups most probably had their differentiation centres at the low elevations of the southern slopes of the southern Carpathians. Based on the actual data set, we cannot exclude the possibility that the central and the south-eastern of these three lineages were, for at least some of the time, a single differentiation centre, or that strong gene flow has occurred between them (Fig. 7). The fourth lineage, composed of populations from the western Carpathians and the northern eastern Carpathians, might have had its glacial differentiation centre in the Carpathian Basin, between the western and the eastern Carpathians (Fig. 7). Interestingly, these areas are centres of endemism of less mobile species groups like myriapods (D. Kime, personal communication). Therefore, the climatic conditions in this region must have been acceptable for the survival of a temperate species like E. medusa. The significantly higher values of the parameters of genetic diversity of these Carpathian Basin populations in comparison with the southern Carpathian populations suggest that the differentiation centre in the Carpathian Basin was at least as big and stable as the ones on the southern Carpathian slopes. Therefore, although living under marginal ecological conditions, the populations in the Carpathian Basin did not suffer more severe genetic bottlenecks than the ones on the southern Carpathian slopes.

The differentiation in Bulgaria has been considerably weaker than in Romania, but nevertheless the genetic structure coincides mostly with the geographical location of the sample stations (Fig. 6). Furthermore, the genetic distances (Nei, 1978) among the distinguished Bulgarian lineages are almost as high as between the two major genetic lineages of Central Europe (Schmitt & Seitz, 2001a). Therefore, we assume that the genetic structure in Bulgaria is also a result of vicariance events, with four centres of differentiation (Fig. 7). The location of these differentiation centres is supported by the distribution of myriapod endemics whose centres of endemism (Stoev, in press) are similar to the postulated differentiation centres of E. medusa.

Based on the available data we cannot decide whether: (1) these vicariance events in Bulgaria only occurred during the second and drier part of the last ice age, with the consequence of a broader distribution during the first half of the Würm ice age, and/or (2) continuous gene flow among these centres reduced the velocity of differentiation, and/or (3) the numbers of individuals per population has been constantly so high that
genetic drift was reduced to a minimum. Based on the genetic distances (Nei, 1978) and on our cluster analysis, past gene flow along the Struma Valley (west of the Pirin Mountains) is unlikely. Therefore, we postulate gene flow east of the Bulgarian high mountain systems (Fig. 7).

CONCLUSION

Erebia medusa has several endemic genetic lineages in Romania and Bulgaria. The strong differentiation between these two countries indicates a long-term separation (at least since the Eem interglacial) of the two main groups by the Danube Valley. The strong differentiation into sublineages in Romania is most probably due to the existence of several glacial differentiation centres, two or more south of the southern Carpathians and one in the eastern part of the Carpathian Basin. The less pronounced differentiation in Bulgaria into sublineages is most probably the product of glacial vicariance too, but maybe only during the second half of the last ice age and/or with constant gene flow among these differentiation centres. The considerably lower genetic diversity in the Romanian populations in comparison with the Bulgarian ones is taken to indicate that the environmental conditions in Romania during the last ice age were considerably worse than further south in the Balkan Peninsula, and that therefore the former went through pronounced genetic bottlenecks.

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