

Are *Polyommatus hispana* and *Polyommatus slovacus* bivoltine *Polyommatus coridon* (Lepidoptera: Lycaenidae)? The discriminatory value of genetics in taxonomy

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Abstract

Taxonomic confusion exists in several sibling species groups. The *Polyommatus coridon* species complex (Chalk Hill Blues) serves as a model group of sibling species in which genetic analyses provide suitable means for taxonomic clarification. We studied the allozyme patterns of the two described bivoltine species of this complex, *Polyommatus hispana* and *Polyommatus slovacus*, and compared them to the two genetic lineages of the univoltine *P. coridon*. *P. hispana* is well distinguished from *P. coridon* (genetic distance: 0.081), and most probably is a sibling species that has evolved during glacial isolation on the Iberian Peninsula. *P. slovacus* is genetically indistinguishable from the eastern, Pontic–Mediterranean lineage of *P. coridon*; therefore we suggest that it represents a local bivoltine population only. Since the spring generation of *P. slovacus* was much less common than the summer generation and showed less genetic diversity, it is probable that uni-/bivoltinism is a dimorphism affecting only part of the whole population. We suggest that the higher genetic diversity of the second generation may be a consequence of gene flow from adjacent single-brooded populations.

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Introduction

The essentialist, static morphospecies concept has been transformed since the times of Linnaeus into the biological species concept based on interspecific reproductive isolation, intraspecific recognition and genetic cohesion. Consequently, the term “species” as a concept, category and taxon has become differentiated

(Mayr 1963; Dobzhansky 1970; Paterson 1985; Templeton 1989; Harrison 1998; Howard and Berlocher 1998; Bock 2004). Many different analytical methods are being applied to the diagnosis of specific differentiation of morphologically rather similar taxa. Among them, interspecific morphological differences in genital structures have been reconsidered and re-evaluated as indicators of reproductive isolation (e.g. Lafontaine and Mikkola 1987; Shapiro and Porter 1989; Mikkola 1992). In addition, chemical and acoustic signals, life cycle traits, and host species specialisations often have been used as tools for species discrimination (e.g. Ragge

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and Reynolds 1984; Ragge 1987; Ingrisch 1995; Quartau et al. 2000; Fischer and Fiedler 2002).

The rapid development of molecular techniques, especially the advances in molecular genetics, provide very useful tools in this field (cf. Loxdale and Lushai 1998). These techniques are widely used to analyse general phylogenetic relationships or phylogeographic structures within species (cf. Hillis and Moritz 1995; Avise 1998; Comes and Kadereit 1998; Howard and Berlocher 1998; Taberlet et al. 1998; Hewitt 1999, 2000, 2001, 2004). However, for invertebrates much fewer examples are known in which these techniques have been used to distinguish between cryptic and/or sibling species pairs. Such examples include the neotropical *Heliconius charithonia* group (Jiggins and Davies 1998), the *Aricia* group (Aagaard et al. 2002), the *Cicada orni* group (Quartau et al. 2000), and aquatic gastropods (Wilke and Pfenninger 2002; Pfenninger et al. 2003). Recent studies support the view that the level of phylogenetic differentiation between or within closely related species based on DNA sequence data can be compared with genetic distances obtained from allozyme studies (e.g. the often discussed *Maculineaalconrebeli* complex; see Als et al. 2004; Bereczki et al. 2005).

The rather diverse genus *Polyommatus* Kluk, 1801 (s.l.), subdivided into several subgenera, is a suitable group for such taxonomic case studies, because many taxa of still unresolved taxonomic status exist in this group (e.g. subgenus *Agrodiaetus*, consisting of over 50 species, many of which are strictly endemic). Moreover, aggregates of sibling species also are characteristic for this genus. The *Polyommatus coridon* complex (Chalk Hill Blues) of the subgenus *Lysandra* represents a very suitable model group of sibling species to test the usefulness of allozyme analysis amidst such taxonomic complexity. This assemblage is composed of several, mostly allopatric species in Europe. *P. coridon* (Poda, 1761) is widely distributed in southern and central Europe and is rather abundant on semi-natural calcareous grasslands; its single generation is on the wing in July and August (Tolman and Lewington 1998). Several authors accept *P. coridon caelestissima* (Verity, 1921) as a separate species that is restricted to a narrow area in central Spain (e.g. Manley and Allcard 1970; Fernández-Rubio 1991). However, this is not well supported by recent genetic analyses (Lelièvre 1992; Marchi et al. 1996). The contrary situation is seen in *P. coridon gennargentii* (Leigheb, 1987) from Sardinia, which might be a separate species due to its strong genetic differentiation from continental *P. coridon* (Marchi et al. 1996). This is also supported by recent studies of preimaginal morphology and the life history of this taxon (Jutzeler et al. 2003). Another univoltine species occurring in central and eastern Spain, *Polyommatus albicans* (Herrich-Schäffer, 1851), is generally accepted in the literature (cf. Tolman and Lewington 1998), albeit

with rather weak support from genetic data (Lelièvre 1992).

Two taxa in the *P. coridon* group differ considerably from all others by being bivoltine. *Polyommatus hispana* (Herrich-Schäffer, 1852) is distributed along the Mediterranean coast from south-eastern Spain to north-western Italy. Its typical habitat is similar to that of *P. coridon*. The spring generation of these butterflies is on the wing from April to June, the second generation from August to October (Tolman and Lewington 1998). Recently, another bivoltine species, *Polyommatus slovacus* Vít'az et al., 1997, was described with a very limited distribution in the dolomitic hills of south-western Slovakia. Its spring generation is on the wing from the second half of May to the middle of June, the second generation at the same time as the univoltine *P. coridon*, from the second half of July to the middle of August. Both generations are supposed to be complete; the adults of the second generation emerge in the laboratory about 20 days after pupation (Vít'az et al. 1997). So far, there is no evidence of hybridisation in the wild between synchronous second-brood *P. slovacus* and univoltine *P. coridon*. However, the spot-like occurrence of the bivoltine *P. slovacus* is surrounded by monovoltine populations of *P. coridon*. The habitat of both types of populations, calcareous rupicolous grassland, also does not show noteworthy differentiation. Therefore, frequent hybridisation in the field appears possible.

Both of the bivoltine species show very few morphological differences from the univoltine *P. coridon*. Therefore, it is important to test their specific status using genetic markers. In the present study, the questions are (i) whether *P. hispana* is a sibling species of *P. coridon* or only represents bivoltine populations of the latter, (ii) whether *P. slovacus* is different from *P. coridon* on the specific level as well, and (iii) whether *P. slovacus* represents remote populations of *P. hispana* in eastern Europe. In addition, an evaluation of the biogeography and evolutionary history of *P. hispana* and *P. slovacus* is performed.

Materials and methods

We sampled the spring generation of *P. hispana* from two populations: 25 males and three females at Sanilhac (Gard, southern France) on 14.V.1998, and 50 males at La Braise (Alpes Maritimes, south-eastern France) on 16.V.1998. For *P. slovacus*, we sampled both generations at the type locality, Lúka nad Váhom in south-western Slovakia: 39 males on 23.V.1997, and 50 males on 10.VIII.1997. We stored the individuals in liquid nitrogen until further analysis. The geographic positions of these sampling sites, and those of additional samples of *P. coridon*, are shown in Fig. 1.

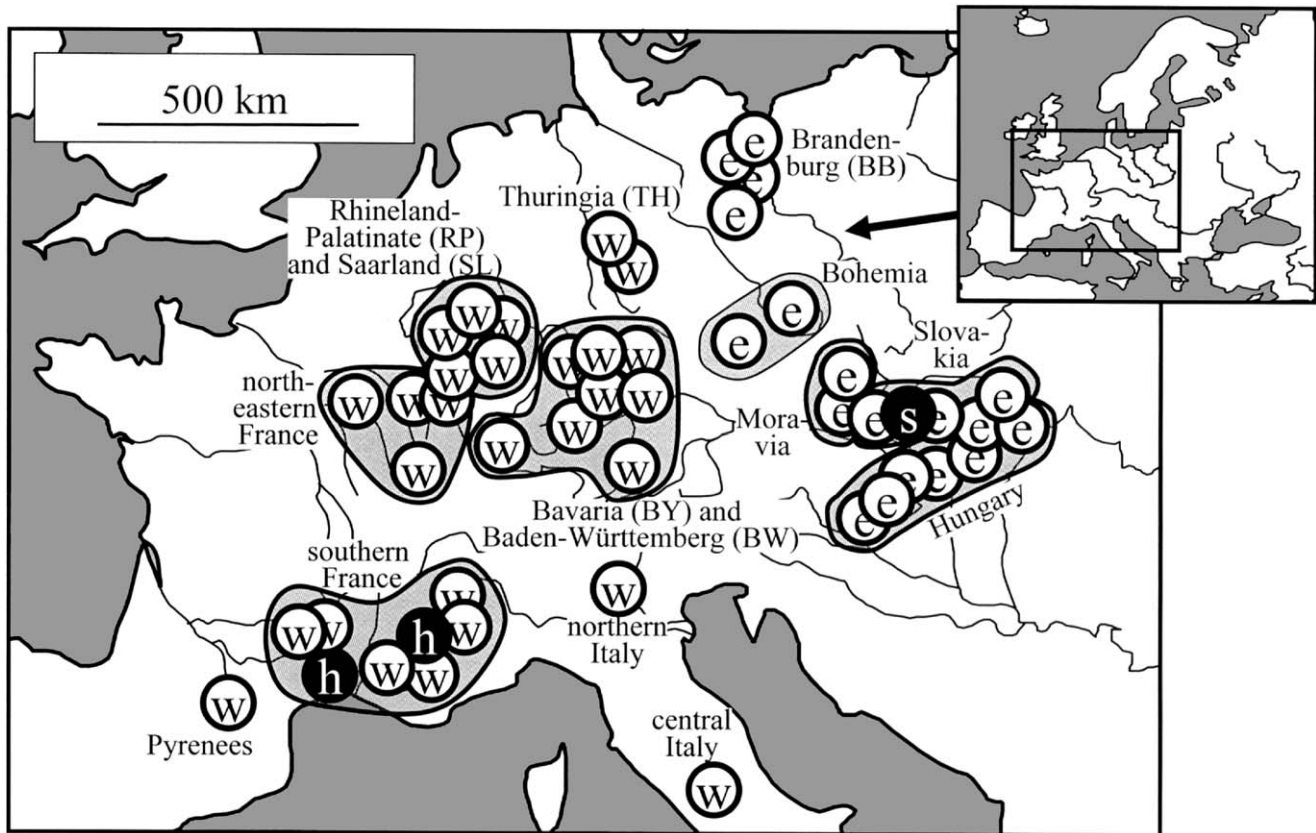


Fig. 1. Geographic distribution of sample sites; e = *Polyommatus coridon*, eastern lineage; h = *P. hispana*; s = *P. slovacus*; w = *P. coridon*, western lineage. Exact locations can be obtained from the corresponding author by request.

Half of the abdomen of each individual was homogenised by ultrasound in Pgm-buffer (Harris and Hopkinson 1978) and centrifuged at 17,000g for 5 min. Electrophoresis was run on cellulose acetate plates (Hebert and Beaton 1993). A total of 17 enzymes representing 20 loci were analysed. Details on loci analysed and electrophoresis conditions are given in Schmitt and Seitz (2001a).

Allele frequencies, F -statistics (Weir and Cockerham 1984) and Nei's (1978) standard genetic distances were calculated by means of the G-Stat package (Siegismund 1993). AMOVA and hierarchical F -statistics were performed using Arlequin 2.000 (Schneider et al. 2000). Hardy–Weinberg equilibrium (Louis and Dempster 1987) and genetic disequilibrium (Weir 1991) were analysed, and exact tests for differentiation (Raymond and Rousset 1995a) were performed with the GENEPOP package (Raymond and Rousset 1995b). Phylogenetic trees using the UPGMA algorithm were constructed with PHYLIP version 3.5.c (Felsenstein 1993). Bootstraps based on 1000 iterations were calculated with the same software.

A comparison with the two major genetic lineages of *P. coridon* was performed, including previously published data on *P. coridon*. We used all 36 populations from a major phylogeographic analysis of this species

(Schmitt and Seitz 2001b). We added supplementary populations from regions close to the samples of *P. hispana* in southern France (Schmitt et al. 2002), and *P. slovacus* in east-central Europe (Schmitt and Seitz 2002a). Thus, a total of 46 *P. coridon* populations were included (see Fig. 1). A population of *P. bellargus* (Rottemburg, 1775) from western Germany was included as outgroup.

Results

All loci showed banding patterns consistent with autosomal inheritance and with known quaternary structures (Richardson et al. 1986). We know from *P. coridon* samples that the 6-Pgdh locus in this species is located on the Z chromosome (Schmitt and Seitz 2001b). Although in butterflies the female sex is the hemizygous one and we mostly studied male individuals (only three females were included), this has no consequences for our study. No significant deviations from the Hardy–Weinberg equilibrium were detected for the populations studied ($P > 0.99$). Linkage disequilibrium between loci was not detected after Bonferroni correction. Hardy–Weinberg proportions and indepen-

Table 1. Four parameters of genetic diversity of the two generations of *P. slovacus* from its type locality Lúka nad Váhom, and two populations of *P. hispana* from southern France; for comparison, means and standard deviations for two lineages of *P. coridon* (data reanalysed from Schmitt and Seitz 2001a, 2002a; Schmitt et al. 2002)

		<i>A</i>	<i>H_e</i> (%)	<i>P₉₅</i> (%)	<i>P_{tot}</i> (%)
<i>P. slovacus</i>	Spring	2.50	17.0	55	85
<i>P. slovacus</i>	Summer	2.90	18.5	60	85
<i>P. hispana</i>	Sanilhac	2.65	27.5	65	75
<i>P. hispana</i>	La Braisse	2.50	26.6	60	65
<i>P. coridon</i>	Western ^a	2.79 ± 0.30	20.5 ± 1.6	52 ± 6	77 ± 9
<i>P. coridon</i>	Eastern ^b	2.61 ± 0.34	19.7 ± 1.5	56 ± 6	74 ± 9

A = mean number of alleles per locus, *H_e* = expected heterozygosity, *P₉₅* = percentage of polymorphic loci with the most common allele not exceeding 95%, *P_{tot}* = total percentage of polymorphic loci.

^aMean of 28 populations of an Adriatic–Mediterranean lineage.

^bMean of 18 populations of a Pontic–Mediterranean lineage.

dence of loci therefore allowed the use of standard methods for further analyses.

Several population-genetic parameters were analysed for the samples: the mean number of alleles per locus (*A*), expected heterozygosity (*H_e*), percentage of polymorphic loci with the most common allele not exceeding 95% (*P₉₅*), and the total percentage of polymorphic loci (*P_{tot}*). All data are given in detail in Table 1 and are compared with data obtained for two major genetic lineages of *P. coridon* (reanalysed data from Schmitt and Seitz 2001a, 2002a; Schmitt et al. 2002). The allelic constitution differed significantly between the two populations of *P. hispana* (exact test: $\chi^2 = 56.8$, $P = 0.004$). No significant difference was detected between the two generations of *P. slovacus* (exact test: $\chi^2 = 36.8$, $P = 0.332$).

The genetic distance (Nei 1978) between the two populations of *P. hispana* was 0.0248. Total genetic variance of *P. hispana* was 2.400 ($P < 0.0001$); 1.6% ($P = 0.0068$) of this total genetic variance was between these two populations. Total variance of *P. slovacus* was 1.600 ($P < 0.0001$); the variance between the two generations was not significant (0.008, $F_{ST} = 0.5\%$, $P = 0.15$). The genetic distance (Nei 1978) between *P. hispana* and *P. slovacus* was 0.0845 (± 0.0068 SD). A hierarchical variance analysis revealed 95.6% of the total variance between populations between these two taxa ($F_{GT} = 19.6\%$, $P < 0.0001$), and 4.4% within taxa ($F_{SG} = 1.1\%$, $P < 0.0001$).

Phenograms were constructed based on the genetic distances (Fig. 2), in which we included 46 populations of *P. coridon* from Slovakia, the Czech Republic, Hungary, Germany, France and Italy, and one *P. bellargus* sample from Idar-Oberstein (western Germany). In these phenograms, the two generations of *P. slovacus* clustered together and formed a monophyletic group with the populations of the Pontic–Mediterranean lineage of *P. coridon*. *P. hispana* populations represented an independent clade (Fig. 2).

Differentiation between the two *P. slovacus* samples and the 46 *P. coridon* samples was significant ($F_{GT} = 3.95\%$, $P < 0.0001$). If only the 18 *P. coridon* populations of the eastern lineage were included, this differentiation decreased strongly ($F_{GT} = 1.44\%$, $P = 0.011$), and lost significance if only populations from Slovakia and the south-eastern Czech Republic were used ($F_{GT} = 0.96\%$, $P = 0.11$).

On the other hand, the two samples of *P. hispana* were well distinguished from the 48 populations of the *P. coridon/slovacus* cluster, and 75.6% of the total variance between populations was between these two groups ($F_{GT} = 15.99\%$, $P < 0.0001$). Including successively less *P. coridon* populations, which were closer to the sampling sites of *P. hispana*, did not notably change this ratio. The whole data set is shown in Table 2. The mean genetic distance (Nei 1978) between *P. hispana* and *P. coridon* was 0.0815 (± 0.0114 SD). This was twice as much as between the two lineages of *P. coridon* (0.0407 ± 0.0102 SD).

We also searched for a spring generation of *P. coridon* at three flight sites within a radius of less than 100 km around Lúka nad Váhom (Hradište and Podhradie, both in western Slovakia, on 30.V.1998; and Klentnice, in south-eastern Moravia, on 01.VI.1997). No spring generation has been observed at these three localities. On 10./11.VIII.1997, *P. coridon* was very common at Hradište (more than a thousand individuals in one place), but relatively scarce in Klentnice and Podhradie (less than a hundred individuals seen over several hours). At Lúka nad Váhom, the spring generation was present during both spring visits (23.V.1997 and 30.V.1998), but was relatively scarce (less than a hundred individuals during more than an hours' stay), while the summer generation was extremely common (far more than a thousand individuals in one place) on 10.VIII.1997.

P. hispana was rather scarce at its more western sampling site (Sanilhac: less than 50 butterflies observed

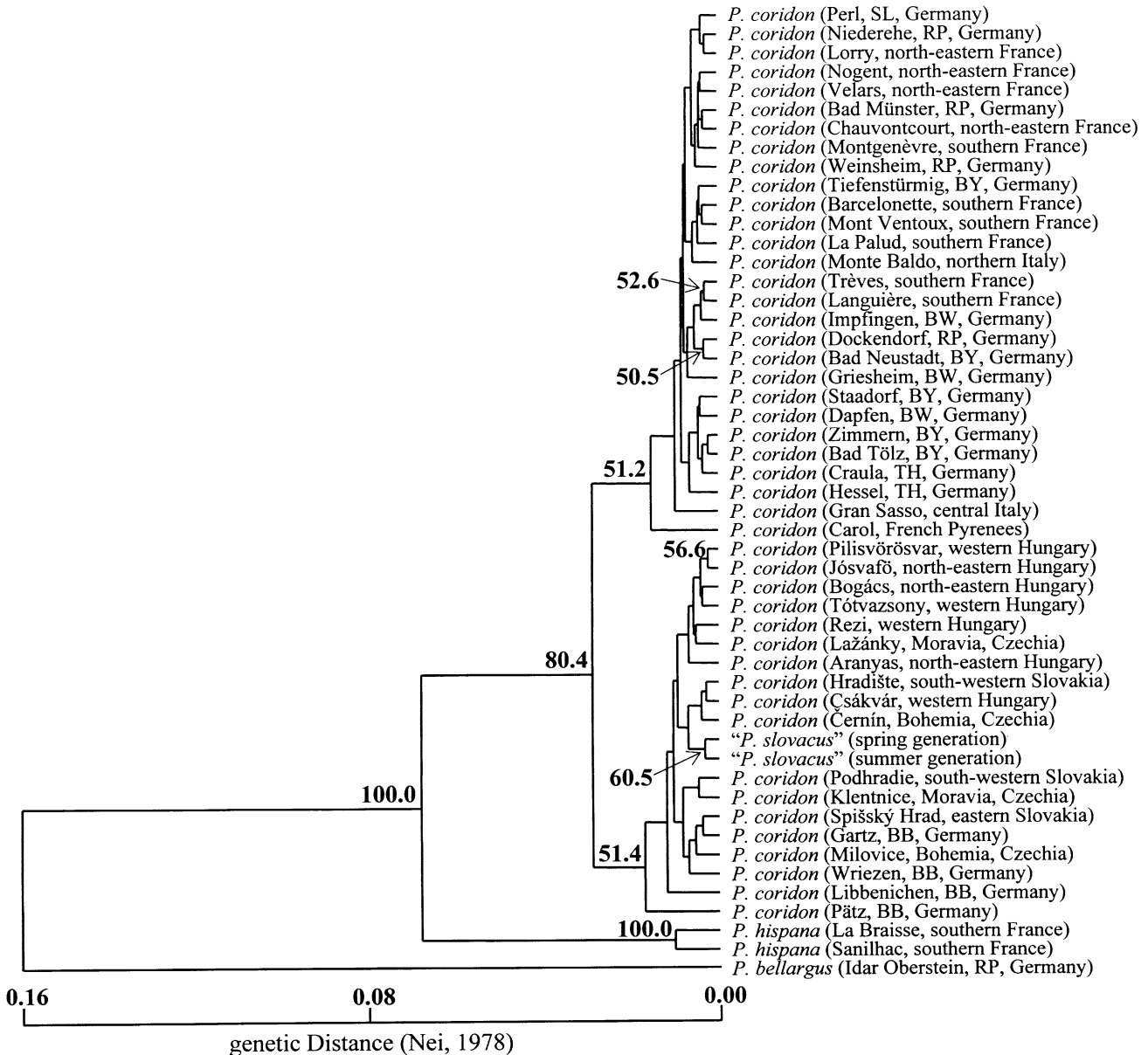


Fig. 2. UPGMA phenogram of genetic distances (Nei 1978) between two populations of *Polyommatus hispana*, two generations of *P. slovacus*, and 46 populations from two genetic lineages of *P. coridon*, with *P. bellargus* included as outgroup; values above branches indicate bootstrap percentages from 1000 iterations. Abbreviations (States in Germany, see Fig. 1): BB = Brandenburg, BW = Baden-Württemberg, BY = Bavaria, RP = Rhineland-Palatinate, SL = Saarland, TH = Thuringia.

during a day of sampling), but extremely common at its more eastern sampling locality (La Braise: far more than a thousand butterflies in one place).

Discussion

The respective total genetic diversities of *P. hispana* and *P. slovacus* were as high as those typically observed

in *P. coridon*, and nearly all values obtained were within the known range of variation of the latter species (see Table 1). Thus, total genetic diversity was considerably higher than in the majority of allozyme studies on butterflies and moths; only a few species with higher genetic diversity are known (cf. Graur 1985; Packer et al. 1998; Schmitt et al. 2002; Wood and Pullin 2002). The percentage of heterozygosity of *P. hispana* was higher even than in *P. coridon*; we are not aware of any work on butterflies reporting higher mean values. Only

Table 2. Hierarchical variance analyses and *F*-statistics between *P. hispana*, *P. slovacus* and *P. coridon*; vapwg = variance among populations within groups, vag = variance among groups

	vapwg	F_{SG}	vag	F_{GT}
Two populations of <i>P. hispana</i> ↔				
48 populations of <i>P. coridon</i> from two major genetic lineages	0.120***	0.0615***	0.370***	0.1599***
28 populations of <i>P. coridon</i> , western lineage	0.040***	0.0213***	0.396***	0.1739***
11 populations of <i>P. coridon</i> , France	0.029***	0.0146***	0.359***	0.1511***
7 populations of <i>P. coridon</i> , southern France	0.036***	0.0171***	0.353***	0.1441***
<i>P. hispana</i> (La Braisse) ↔				
4 populations of <i>P. coridon</i> , southern France east of Rhône	0.012*	0.0057*	0.347***	0.1422***
<i>P. hispana</i> (Sanilhac) ↔				
2 populations of <i>P. coridon</i> , southern Massif Central	0 ^{ns}	0 ^{ns}	0.461***	0.1816***
Two generations of <i>P. slovacus</i> ↔				
48 populations of <i>P. coridon</i> from two major genetic lineages	0.114***	0.0596***	0.079**	0.0395**
18 populations of <i>P. coridon</i> , eastern lineage	0.051***	0.0276***	0.027*	0.0144*
5 populations of <i>P. coridon</i> , Slovakia and Moravia	0.043***	0.0237***	0.018 ^{ns}	0.0096 ^{ns}
2 populations of <i>P. coridon</i> , south-western Slovakia	0.031***	0.0185***	0 ^{ns}	0 ^{ns}

^{ns}*P* > 0.05; **P* < 0.05; ***P* < 0.005; ****P* < 0.0001.

two species of noctuid moths (*Heliothis virescens* and *H. zea*) are on record with higher heterozygosities exceeding 30% (Sluss et al. 1978).

Polyommatus hispana* – an Atlantic–Mediterranean sibling of *P. coridon

The genetic distance between *P. hispana* and *P. coridon* is remarkable and suggests a long period of evolutionary separation of both taxa. The fact that *P. hispana* is similarly differentiated from nearby and distant *P. coridon* populations makes environmental selection unlikely as a trigger for this differentiation. Furthermore, even major introgression of *P. hispana* into *P. coridon*, or vice versa, can be largely excluded. In butterflies and moths, such strong genetic differentiation is known only from complexes with at least subspecific or specific structuring (e.g. Stock and Castroville 1981; Porter and Geiger 1988; Porter et al. 1995; Pratt 1994; Britten et al. 1995; Marchi et al. 1996; Jiggins and Davies 1998; Wiemers 1998; Schmitt and Seitz 2001c). Only half of this distance was observed between two genetic lineages of *P. coridon* described as different subspecies or subspecies complexes (Schmitt and Seitz 2001a), which most probably evolved during Würm-glacial isolation in the Adriatic- and the Pontic–Mediterranean refugium, respectively (Schmitt and Seitz 2001b). It is therefore most likely that *P. hispana* is an Iberian sibling species in its own right, and that it differentiated to species level allopatrically during glacial isolation on the Iberian Peninsula, due to random genetic drift. Such a differentiation of a common ancestor into three major genetic lineages within the three large southern European peninsulas is a frequently observed phylogeographical pattern (cf.

Comes and Kadereit 1998; Taberlet et al. 1998; Hewitt 1999). A refugium of *P. hispana* on the south-eastern Mediterranean coast of Spain seems most probable, given the species' current distribution pattern (Tolman and Lewington 1998). This evolutionary core area was also suggested by Varga (1977).

It could be argued that the stronger differentiation of *P. hispana* in relation to the two *P. coridon* lineages is because (i) the molecular clock (i.e. the speed of evolution) runs faster in *P. hispana* than in *P. coridon*, probably due to the two generations per year; and/or that (ii) differentiation between *P. hispana* and *P. coridon* started earlier than within *P. coridon* itself, with the first split possibly taking place during the Riss glaciation, thereby allowing more time for their evolution; and/or that (iii) the isolation of the Atlantic–Mediterranean refugium was more stringent than between the Adriatic- and the Pontic–Mediterranean ones (and thus better conserved evolutionary steps in Iberia).

The more eastern population of *P. hispana* showed minor genetic diversity for all analysed parameters compared to the more western one. This might be the result of an iterative sampling effect (i.e. random genetic erosion) during the eastward postglacial expansion along the Mediterranean coast of France, most probably due to repeated founder events (cf. Hoelzel et al. 2002). Similar phenomena of loss of diversity during the postglacial expansion process were also observed in the Adriatic- and the Pontic–Mediterranean lineage of *P. coridon*, respectively (Schmitt and Seitz 2001b). This effect is well known from many animals and plants (cf. Hewitt 1996; Comes and Kadereit 1998) and was postulated already by Reinig (1938).

This hypothesis is corroborated by the fact that *P. hispana* in the more eastern population was very abundant, whereas the more western population had a

rather low density. For *P. coridon*, it has been shown on a regional scale that big populations generally possess higher genetic diversity than small ones (Schmitt and Seitz 2002b), a phenomenon that has been observed frequently in plants and animals (e.g. Billington 1991; Buza et al. 2000; Hudson et al. 2000; Jäggi et al. 2000; Madsen et al. 2000). However, we obtained the opposite result, even with fewer individuals analysed from the more western than from the more eastern population.

Polyommatus* “slovacus” – a local bivoltine race of the Pontic–Mediterranean lineage of *P. coridon

The status of *P. “slovacus”* is rather different from that of *P. hispana*. In our analysis, both generations were not significantly different from the univoltine *P. coridon* populations from Slovakia and Moravia, which formed one monophyletic group with other populations from Hungary, the Czech Republic and north-eastern Germany (Schmitt and Seitz 2001a). Thus, no separate refugium for *P. “slovacus”* can be suggested. The refugium of the eastern *P. coridon* lineage has been located further south, probably near the Adriatic coasts of the Balkans (Schmitt and Seitz 2001b), so that the region of Lúka nad Váhom could be colonised only by postglacial range expansion. Thus, the evolution of bivoltinism in south-western Slovakia appears to be a postglacial phenomenon, because *P. “slovacus”* is known only from this region (Vít'az et al. 1997).

If so, the differentiation should have taken place in sympatry or local micro-allopatry with *P. coridon*, maybe due to only one or a small number of mutations within one or a rather limited number of individuals. This hypothesis is supported by the fact that the habitat of the bivoltine *P. “slovacus”* is island-like, dolomitic, hilly rupicolous grassland with rather shallow, sceleritic soils. This means that such sites were greatly resistant to postglacial reforestation even during humid climatic periods, so that they possibly represent natural habitats of *P. coridon* that have persisted since the climate rewarmed.

However, such mutation(s) could have led to speciation due to incompatibility with *P. coridon*, and in this case we would expect a strong genetic bottleneck, as shown, for example, during the evolution of *P. coridon gennargentii* in Sardinia (Marchi et al. 1996). This island taxon showed a dramatically reduced level of genetic diversity for all analysed parameters in comparison to continental populations. In contrast, the summer generation of *P. “slovacus”* showed a genetic diversity slightly higher than other *P. coridon* populations in surrounding Slovakia, Moravia and Hungary.

The spring generation in both years was much more scarce than the summer generation. Therefore we suppose that the phenomenon of bivoltinism of *P.*

coridon is the result of local genetic polymorphism, with a more or less constant proportion of the whole population being bivoltine. However, even the spring generation showed a genetic diversity not differing remarkably from smaller *P. coridon* populations of this region or from more northern localities (Schmitt and Seitz 2002a; see Table 1). In addition, the higher diversity of the summer generation can be explained by gene flow from surrounding synchronous, monovoltine *P. coridon* populations.

This high level of genetic diversity and the low level of genetic differentiation from the adjacent univoltine *P. coridon* populations make the hypothesis likely that the individuals flying in the second half of July and August simply represent a mixture of the phenotypically inseparable second-brood *P. “slovacus”* and synchronous single-generation *P. coridon*, with massive gene flow between them. This might also explain the reduced genetic diversity of the spring generation in comparison to the summer generation.

Consequently, we argue that *P. “slovacus”* is not a separate species. This is also supported by a comparison of the morphology of the type material illustrated in Vít'az et al. (1997) with the *P. “slovacus”* and *P. coridon* material collected by us. In contrast, the two genetic lineages of *P. coridon* (conforming to Schmitt and Seitz 2001a) differ morphologically: they are well distinguished by the width of the dark subterminal fringe stripe (T. Schmitt, unpubl. data). Regarding this feature, *P. “slovacus”* clustered clearly within the Pontic–Mediterranean lineage. Also, the whitish fringe circles of the forewing were missing, as is normal in the eastern lineage.

However, the Lúka nad Váhom individuals were smaller than other *P. coridon*, possibly due to a reduced larval life span. Such a phenomenon has been studied in detail in the life history of *Lycæna hippothoe*. The south-western Hungarian lowland populations of this species are known to produce two generations per year, and were described as separate subspecies (Szabó 1956; Fazekas and Balazs 1979), whereas *L. hippothoe* has only one generation over most of its range (Tolman and Lewington 1998). This life history difference was well supported by rearings in common environments (Fischer and Fiedler 2002). Interestingly, the bivoltine lineage showed a reduced larval life span, an enhanced larval growth rate and a reduced adult weight, most probably as an evolutionary adaptation to bivoltinism (Fischer and Fiedler 2002), and thus might reflect the same phenomenon as in *P. “slovacus”*.

Bivoltinism of normally univoltine Lepidoptera was also observed for several other species. Some burnet moths show highly interesting life histories: *Zygaena trifolii* always has a single generation in central Europe (even in common environment rearings), whereas populations from south-western Europe have two or

more generations, in the wild as well as in common environment rearings (Wipking 1988). *Zygaena fausta* in south-eastern Spain and *Z. transalpina (hippocrepidis)* in south-western France occur in two generations per year in geographically restricted regions (Fernández-Rubio 1990; Naumann et al. 1999). Also, the widely distributed *Zygaena filipendulae* produces two generations per year in many parts of south-central Europe, e.g. parts of northern Italy and the lowlands of Hungary (*Z. Varga*, pers. observ.). Apparently, genetic polymorphisms also evolved in these species and survived in some limited areas, possibly even close to the respective place at which that evolution had occurred, as most probably is the case in *P. "slovacus"*.

Since plurivoltinism has been considered a plesiomorphic feature in *Polyommatus* species (Schurian 1989), this local bivoltinism of *P. coridon* was conserved or re-evolved in this local strain. As all the other known *P. coridon* populations of the eastern lineage are univoltine, even in regions with generally higher temperatures and longer vegetation periods than in Slovakia, the bivoltinism of *P. "slovacus"* cannot be interpreted as an adaptation to particularly warm and favourable climatic conditions. Whether this life history trait is advantageous and will spread, or whether it is less advantageous and finally will disappear, is an open question.

Conservation implications

P. hispana, the western and the eastern *P. coridon* lineage represent three well-distinguished units. As evolutionarily significant units (ESUs), they merit independent conservation efforts. Among these three taxa *P. hispana* is the unit with the smallest distribution area (Kudrna 2002), and therefore special conservation responsibility for this taxon has to be attributed to Spain and France. Both ESUs of *P. coridon* are so widespread that the conservation responsibility for either of these two taxa is shared by many European countries. Nevertheless, as *P. coridon* is one of the best character species of semi-natural calcareous grasslands (van Swaay 2002), conservation measures for *P. coridon* directly help keep their habitats protected by the habitat directive of the European Union.

The assessment of the conservation value of the bivoltine *P. "slovacus"* is more difficult. From the population-genetic point of view, there is nothing extraordinary in these individuals that would justify special conservation efforts. However, the character of bivoltinism opens some novel evolutionary possibilities. This might justify even the classification as a separate ESU; at least, this bivoltine strain of *P. coridon* has to be considered as a special management unit (MU) with

high priority for conservation. Also, the habitat of this population ranks among the habitat types requiring special conservation measures as well (Habitat Directive, Annex I: 6190 Rupicolous pannonic grasslands, *Stipo-Festucetalia pallentis*), and represent one of the northernmost, isolated sites of dolomitic rupicolous grasslands of the Pannonian type.

Conclusions

Although *P. hispana* and *P. slovacus* initially appeared as quite similar entities, our analysis revealed rather different evolutionary histories. In the case of *P. hispana*, allopatric speciation most probably took place during a long-lasting process of isolation. On the other hand, *P. slovacus* was described as a species, which might have evolved in sympatry with *P. coridon* during a rather short time period. However, our allozyme analysis could not support this latter hypothesis, and the bivoltine population from Lúka nad Váhom may be seen as a local race of *P. coridon* with a peculiar atavism of bivoltinism. Thus, our study demonstrates the high discriminatory value of allozyme analyses for the resolution of evolution in sibling-species questions.

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