



Dispersal, fragmentation, and isolation shape the phylogeography of the European lineages of *Polyommatus (Agrodiaetus) ripartii* (Lepidoptera: Lycaenidae)

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Polyommatus ripartii is a biogeographically and taxonomically poorly understood species of butterfly with a scattered distribution in Europe. Recently, it has been shown that this species includes several European endemic and localized taxa (*galloi*, *exuberans*, *agenjoi*) that were previously considered species and even protected, a result that poses further questions about the processes that led to its current distribution. We analysed mitochondrial DNA and the morphology of *P. ripartii* specimens to study the phylogeography of European populations. Three genetically differentiated but apparently synmorphic lineages occur in Europe that could be considered evolutionarily significant units for conservation. Their strongly fragmented and counterintuitive distribution seems to be the result of multiple range expansions and contractions along Pleistocene climatic oscillations. Remarkably, based on the 79 specimens studied, these genetic lineages do not seem to extensively coexist in the distributional mosaic, a phenomenon most evident in the Iberian Peninsula. One of the important gaps in the European distribution of *P. ripartii* is reduced by the discovery of new Croatian populations, which also facilitate a better understanding of the biogeography of the species. © 2013 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2013, 109, 817–829.

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INTRODUCTION

The butterfly subgenus *Agrodiaetus* is distributed throughout the Western Palaearctic and Central Asia. Sexes are often dimorphic, with females usually brown in colour, and with males displaying colours such as white, silver, blue, violet, or brown on the dorsal part of the wings, which is probably a signature of the reinforcement of pre-zygotic isolation (Lukhtanov *et al.*, 2005). *Agrodiaetus* has gone through an explosive radiation, with more than 120

species appearing during the last three million years (Eckweiler & Häuser, 1997; Kandul *et al.*, 2004; Kandul, Lukhtanov & Pierce, 2007; Talavera *et al.*, 2013). Such extreme diversification has been accompanied by an impressive diversity of karyotypes, ranging from $n = 10$ to $n = 134$, and placing *Agrodiaetus* among the most striking examples of chromosome number variation documented (Lesse, 1960; Lukhtanov & Dantchenko, 2002).

It is worth noting that Lepidoptera chromosomes are holocentric, so that holokinetic activity is not restricted to a single site (the centromere), but is spread across the whole (or large parts) of the chromosome (Wolf, Novak & Marec, 1997; Lukhtanov &

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Dantchenko, 2002). This property is likely to be a factor favouring karyotype diversity within this genus through processes of fusion and/or fission (Lorković, 1990). Therefore, butterflies of the subgenus *Agrodiaetus* represent an excellent system to study various aspects of evolution and speciation in action. However, in contrast to the extreme karyotypic variability, these butterflies display few reliable morphological characters that allow species discrimination, rendering them a particularly difficult taxonomic group (e.g. Forster, 1956, 1960, 1961; Wiemers, 2003). This is especially true for species of the 'brown type', where both sexes have uniformly brown wing upper-sides. As a consequence, species delimitations in *Agrodiaetus* have relied heavily on differences in chromosomal complements (e.g. Lesse, 1960; Munguira, Martín & Pérez-Valiente, 1995) and, more recently, on molecular data (e.g. Wiemers, 2003; Kandul *et al.*, 2004; Lukhtanov, Vila & Kandul, 2006; Vila *et al.*, 2010). This is probably the reason why a taxonomic group with such potential for evolutionary studies traditionally lacks the detailed distributional and biogeographic studies that complement them.

Although the centre of *Agrodiaetus* diversity is in the Caucasus, Asia Minor, and Iran (Wiemers, 2003), a substantial number of taxa are also known to occur in the Mediterranean part of Europe (Hesselbarth, Oorschot & Wagener, 1995; Eckweiler & Häuser, 1997). The group is taxonomically problematic in this area, and this is reflected in the various numbers of species (ranging from 11 to 22) reported for Europe by different authors (De Prins & Iversen, 1996; Dennis, 1997; Kudrna, 2002; Dennis *et al.*, 2008; Kudrna *et al.*, 2011). A recent study that focused on Western Europe has revealed considerable taxonomic oversplitting in the number of *Agrodiaetus* species (Vila *et al.*, 2010). Although not fully documented, a similar situation seems to exist in the Balkans, with at least two taxa, namely *eleniae* (Coutsis & De Prins, 2007) and *nephohiphtamenos* (Brown & Coutsis, 1978), suspected to represent synonyms of other species (Wiemers, 2003; Kudrna *et al.*, 2011). As several taxa are considered endemics with highly restricted distributions, taxonomic uncertainty in European *Agrodiaetus* can have important implications for nature conservation, as well as for biogeographic inferences. Thus, it is highly recommended that newly discovered populations are thoroughly examined in order to ensure correct species-level identification. Ideally, this should be done by combining multiple sources of data, such as chromosome number, DNA sequences, and/or morphology.

Polyommatus (*Agrodiaetus*) *ripartii* (Freyer, 1830) is a species of the 'brown type', ranging from Spain in the west to Mongolia in the east, but with a fragmented distribution and scattered populations across this range that probably represent a relict distribu-

tion. A recent study based on molecular and karyological data (Vila *et al.*, 2010) recognized three subspecies of *P. ripartii*: the nominative subspecies *Polyommatus ripartii ripartii* (Freyer, 1830), which is widespread from western Europe to eastern Kazakhstan, *Polyommatus ripartii colemani* (Lukhtanov & Dantchenko, 2002) (apparently restricted to Central Asia), and *Polyommatus ripartii paralcestis* (Forster, 1960) (occurring in Asia Minor and Armenia). In Europe, *P. r. ripartii* displays a disjunct distribution between Western Europe (northern Spain, southern France, parts of Italy) and eastern Europe (southern Poland, southern Serbia, western and southern Bulgaria, Republic of Macedonia, Albania, and Greece) (Vila *et al.*, 2010; Kudrna *et al.*, 2011). Very recently, several localized taxa previously considered as species endemic to Europe have been shown to actually represent populations of *P. ripartii* (Vila *et al.*, 2010). These were *gallois* (Balletto & Toso, 1979), representing a very isolated population in southern Italy (Calabria), *exuberans* (Verity, 1926), occurring in north-western Italy (Susa Valley), and *agenjoi* (Forster, 1965), present in north-eastern Spain (Catalonia). Although these recent findings have improved the distribution of *P. ripartii*, the distribution gap between western and eastern Europe is still substantial. The species has also been recently reported from Croatia (Koren, 2010) (Fig. 1). Its presence there is important from a biogeographical point of view as it would considerably reduce the distributional gap between western Europe and the Balkans; however, the record was not unambiguously documented, as it was based on a single male specimen and relied exclusively on external morphology.

All three subspecies of *P. ripartii* seem to have a haploid chromosome number of $n = 90$, which in Europe is shared with the externally similar *Polyommatus fabressei* (Oberthür, 1910) and *Polyommatus violetae* (Gómez-Bustillo, Expósito & Martínez, 1979) (e.g. Lesse, 1960; Munguira *et al.*, 1995; Lukhtanov & Dantchenko, 2002; Vila *et al.*, 2010). Because of the scattered and incompletely understood distribution, as well as taxonomic confusion, *P. ripartii* represents an interesting species from both a biogeographical and taxonomical point of view. In this article we study material from the main European populations of this species to document genetic structure and to reconstruct the colonization of Europe by this species. Additionally, we describe a new population from Croatia that fills an important distributional gap and provides insight into the complex phylogeography of *P. r. ripartii*. We also employ male genitalia and wing morphometry in order to assess the usefulness of these characters for the identification of the species, and to explore potential differences among genetic lineages.

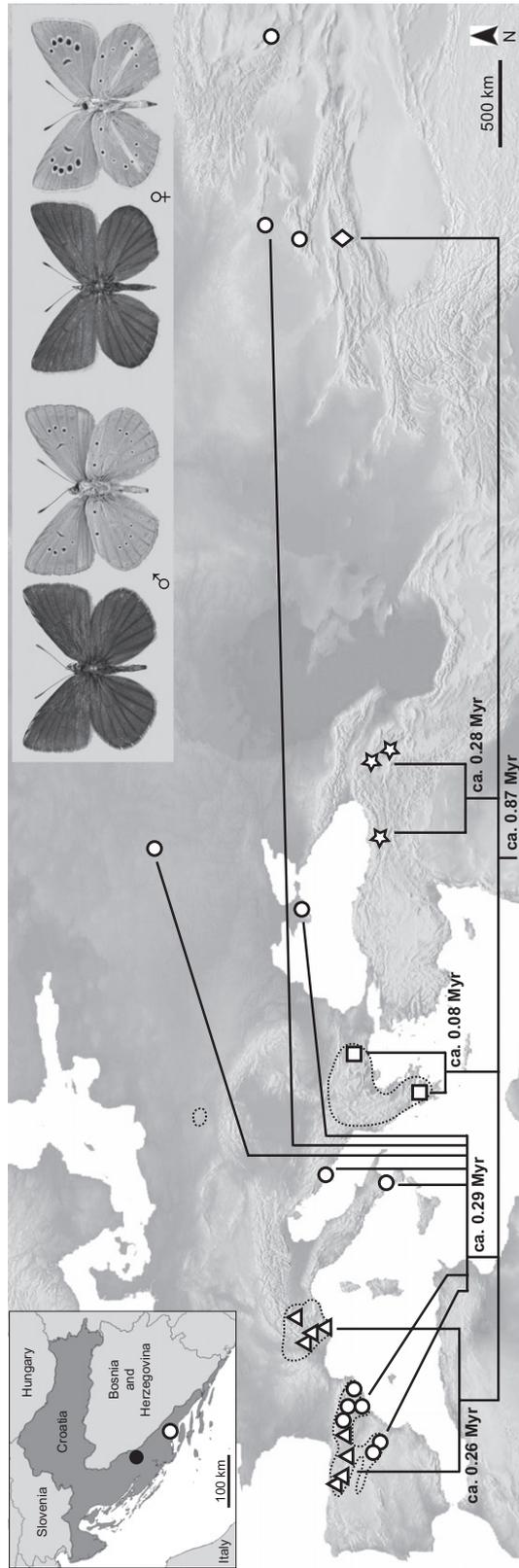


Figure 1. Distribution, sampling localities, and phylogeographic inference for *Polyommatus ripartii*. The general distribution of the species is based on records that are less than 40 years old. Symbols correspond to the main genetic lineages identified: Δ , West European; \square , Eurasian; \circ , Balkans; \star , subspecies *parvalcestis*; \diamond , subspecies *colemanni*. The most recent common ancestor of the *P. ripartii* lineages dates back to the late Pleistocene, c. 0.87 Mya. In Europe, as a consequence of Pleistocene glaciations, the species generated the currently observed mosaic of three genetic lineages that appear to exclude each other. The West European and Balkan lineages probably originated in Europe, whereas the Eurasian lineage is likely to have appeared in central Asia and subsequently colonized Europe. The map in the upper left corner details the location of the newly discovered Croatian population (\circ), as well as the position of a previous record by Koren (2010) (\bullet). The upper right corner illustrates the dorsal and ventral parts of both sexes of *P. ripartii*.

MATERIAL AND METHODS

TAXON SAMPLING

For molecular analyses, we examined 112 *Polyommatus* specimens, 55 of which were mined from GenBank, mainly from the studies of Wiemers (2003), Wiemers & Fiedler (2007), Wiemers, Keller & Wolf (2009), and Vila *et al.* (2010). More precisely, we used the data set for *COI* and *COII* from 45 specimens of the *P. dolus* and *P. admetus* species groups from Vila *et al.* (2010), plus all *COI* sequences of *P. ripartii*, *Polyommatus admetus* (Esper, 1783), and *Polyommatus nephohiptamenos* (Brown & Coutsis, 1978) available in GenBank. The other 57 sequences were sequenced for this study, and included 54 specimens of *P. ripartii* (27 from Spain, 12 from Croatia, seven from Bulgaria, six from France, and two from Armenia), as well as two *P. admetus* (Bulgaria) and one *P. nephohiptamenos* (Bulgaria). *Polyommatus damon* ([Schiffmüller], 1775) was used as the out-group. The taxon *demavendi* (Pfeiffer, 1938) was not included in the analyses because specimens with unambiguous species identification and precise chromosome number were not available (see also Vila *et al.*, 2010).

This resulted in a matrix of *COI* and/or *COII* sequences corresponding to 112 specimens (Table S1). Our data set includes 79 *P. ripartii* specimens, and represents well the main areas of distribution of this taxon in Europe (Fig. 1). Among these are 12 Croatian specimens that considerably reduce the distribution gap between the western and eastern parts of the range of the species. The only isolated European population not sampled was Poland, for which no material was available.

All 57 individuals sequenced for this study are stored in the Butterfly Diversity and Evolution Lab, at the Institut de Biologia Evolutiva (CSIC-Universitat Pompeu Fabra), Barcelona, Spain. The only exception is the Croatian specimen RVcoll.09-V363, which is stored in Märten Nilsson's private collection.

For morphological analyses we used all sequenced males (43 specimens) for which both genitalia and wing vouchers were available.

DNA EXTRACTION AND SEQUENCING

Total genomic DNA was extracted using Chelex 100 resin, 100–200 mesh, sodium form (Bio-Rad), under the following protocol: one leg was removed and introduced into 100 µL of Chelex 10% and 5 µL of Proteinase K (20 mg mL⁻¹) was added. The samples were incubated overnight at 55 °C and were subsequently incubated at 100 °C for 15 min. Samples were then centrifuged for 10 s at 845 g.

A 658-bp fragment at the 5' end of the mitochondrial gene *COI* was amplified by polymerase chain reaction using the primers LepF1 (5'-ATTCAACC AATCATAAAGATATTGG-3') and LepR1 (5'-TAA ACTTCTGGATGTCCAAAAAATCA-3') (Hebert *et al.*, 2004). Double-stranded DNA was amplified in 25-µL reactions containing 14.4 µL autoclaved Milli-Q water, 5 µL 5X buffer, 2 µL 25 mM MgCl₂, 0.5 µL 10 mM dNTPs, 0.5 µL of each primer (10 µM), 0.1 µL Taq DNA Polymerase (Promega, 5 U µL⁻¹) and 2 µL of extracted DNA. The typical thermal cycling profile was: first denaturation at 92 °C for 60 s, followed by five cycles of 92 °C for 15 s, 48 °C for 45 s, and 62 °C for 150 s, and then by 35 cycles of 92 °C for 15 s, 52 °C for 45 s, and 62 °C for 150 s, with a final extension at 62 °C for 420 s. PCR products were purified and sequenced by Macrogen Inc. Sequences were edited and aligned using GENEIOUS PRO 4.7.5, created by Biomatters (<http://www.geneious.com/>).

Fifteen of the 57 novel sequences generated by this study have been obtained at the Biodiversity Institute of Ontario, Canada. In this case, a glass fibre protocol (Ivanova, deWaard & Hebert, 2006) was employed to extract DNA, and polymerase chain reactions (PCRs) and DNA sequencing were carried out following the standard DNA barcoding procedures for Lepidoptera (deWaard *et al.*, 2008). Sequences generated as a result of this study were submitted to GenBank (see Table S1 for accession numbers).

PHYLOGENETIC ANALYSES AND DATING OF PHYLOGENETIC EVENTS

The final alignment used for phylogenetic analyses included 112 sequences and was 2174 bp long, consisting of concatenated sequences of *COI* and *COII*. Phylogenetic relationships were inferred using Bayesian inference (BI). Both BI analyses and the estimation of node ages were run in BEAST 1.6.2 (Drummond & Rambaut, 2007) with the data set partitioned by gene. Substitution models used for each partition were chosen according to the Akaike's information criterion (AIC) values obtained in JMOD-ELTEST 0.1 (Posada, 2008), and were GTR + I + G for *COI* and HKY + I + G for *COII*. Base frequencies were estimated, six gamma rate categories were selected and a randomly generated initial tree was used. As previous studies have shown that *P. damon* is the most basal species of the subgenus *Agrodiaetus* (Kandul *et al.*, 2004; Wiemers *et al.*, 2009; Talavera *et al.*, 2013), we enforced the monophyly of the *P. dolus* + *P. admetus* clade so that *P. damon* is recovered as sister to the rest.

Following the methodology used by Vila *et al.* (2010), we defined priors for the age of the root node (*P. damon*) and another node within the *P. dolus*

species group (Fig. 2). Mean uncorrected pairwise distances for the taxa defined by these two nodes were calculated with MEGA 5 (Tamura *et al.*, 2011). Subsequently, rough estimates of ages were obtained by applying two different molecular clocks with published substitution rates to these pairwise distances: 1.5% uncorrected pairwise distance per million years estimated for various invertebrates (Quek *et al.*, 2004), and 2.3% estimated for the entire mitochondrial genome of several arthropods (Brower, 1994). A normal prior distribution was used in both cases, centred on the mean of ages obtained with the two substitution rates, and the standard deviation was tuned so that the 95% confidence interval of the posterior density coincided with the ages obtained with each of the rates. Parameters were estimated using two independent runs of ten million generations each, and convergence was checked using the program TRACER 1.5.

HAPLOTYPE NETWORK

Unique haplotypes of *P. ripartii* were obtained using the program TCS 1.21 (Clement, Posada & Crandall, 2000) on a 624-bp alignment consisting of 67 specimens. This data set was obtained by excluding short or ambiguous sequences from the total 79 *P. ripartii* included in the study. Although a minimum quantity of data is lost, the final alignment includes only sequences of equal length and is completely free of ambiguities. As the focus of our study was Europe and available material outside this region was limited (and some sequences were below the 624-bp threshold), the genetically diverged Asian subspecies *paralcestis* and *colemanni* (six specimens in total) were not included in the analysis. In order to examine relationships among haplotypes, a maximum parsimony haplotype network was constructed using TCS 1.21, with a 95% connection limit. The network presented two loops, which were broken according to frequency and geographic criteria (Excoffier & Langaney, 1989).

GENITALIA AND WING MORPHOMETRY

The morphometric analyses included 43 males (41 *P. ripartii*, one *P. nephohiptamenos*, and one *P. admetus*) available for the examination of genitalia among the specimens sequenced (Table S2). Male genitalia were prepared according to the following protocol: maceration for 10 min at 95 °C in 10% potassium hydroxide, dissection and cleaning under a stereomicroscope, and storage in 0.5-mL tubes with glycerine. The genitalia were slightly pressed under a cover slip and were photographed in a thin layer of 70% ethanol under a Carl Zeiss Stemi 2000-C stereomicroscope equipped with a DeltaPix Invenio 3S digital camera.

Measurements of the valva length were performed based on the digital photographs by using AXIOVISION. The valva length was measured as the distance between its proximal and distal ends, where the distal end has been considered the valva apex, without taking into account the dorsal flap (Fig. S1A). We used this approach in order to minimize measurement error, as the position of the dorsal flap can vary according to genitalia preparation and the pressure applied to the coverslip. The width of the forewing was used as a proxy for specimen size, and was measured as the distance between the intersection points of the wing margin and veins v1 and v7 (Fig. S1B). In addition, forewing length was measured for a subset of 31 specimens as the remainder had the base of the wings truncated because of the ethanol preservation protocol. Forewing length was measured from the wing base to its apex (Fig. S1C). This measurement was performed in order to allow comparisons with previously published data (Kolev, 1994, 2005; Kolev & De Prins, 1995).

RESULTS

MOLECULAR DATA

The Bayesian ultrametric tree based on mitochondrial DNA recovered the monophyly of *P. dolus* and *P. admetus* species groups with good support (Fig. 2), in accordance with previous results (Wiemers, 2003; Kandul *et al.*, 2004, 2007; Vila *et al.*, 2010). Relationships and supports within the *A. dolus* clade were very similar to the results of Vila *et al.* (2010). The three subspecies of *P. ripartii* provisionally proposed by Vila *et al.* (2010) were genetically distinct, but the relationships among them were not fully resolved, and the monophyly of *P. ripartii* was not supported because of the presence of *P. khorasanensis* (Carbonell, 2001), *P. admetus*, and *P. nephohiptamenos* specimens. However, our results showed that three well-differentiated and supported clades exist within *P. r. ripartii*: one clade exclusively included specimens from the Balkans (called here the Balkan clade); another included specimens from northern Spain (excluding the north-east), south-eastern France, and north-western Italy (called here the West European clade); and the third clade included specimens from a broad geographic area, ranging from north-eastern Spain in the west to eastern Kazakhstan and Mongolia in the east (called here the Eurasian clade). The situation in northern Spain is particularly interesting, as the current data indicate that specimens of the West European and the Eurasian clades are separated by only ~60 km in the province of Huesca, Aragón, north-eastern Spain (Fig. 1). All 12 specimens of the newly discovered

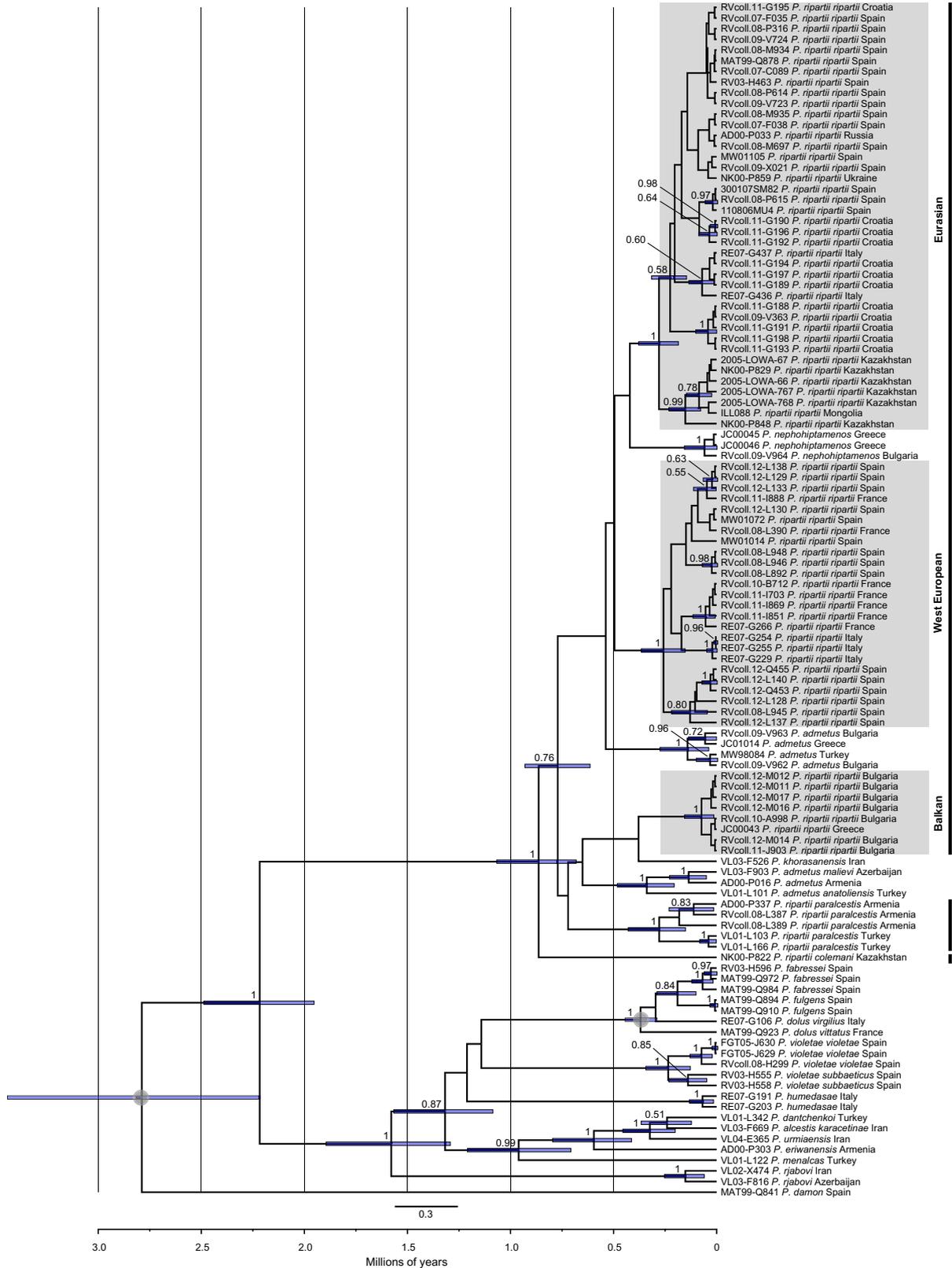


Figure 2. Bayesian ultrametric tree derived with BEAST 1.6.2, based on cytochrome *c* oxidase subunit I (*COI*) and subunit II (*COII*) sequences. The tree was calibrated at two nodes (indicated by grey dots), based on 1.5 and 2.3% uncorrected pairwise distance per million years substitution rates estimated for mitochondrial DNA in Arthropoda. Node bars represent 95% highest posterior density for age estimations. Bayesian posterior probabilities higher than 0.5 are displayed above recovered nodes. The three subspecies of *P. ripartii* recognized by Vila *et al.* (2010) are indicated by black vertical bars, whereas the three main clades of *P. ripartii* occurring in Europe are highlighted in grey.

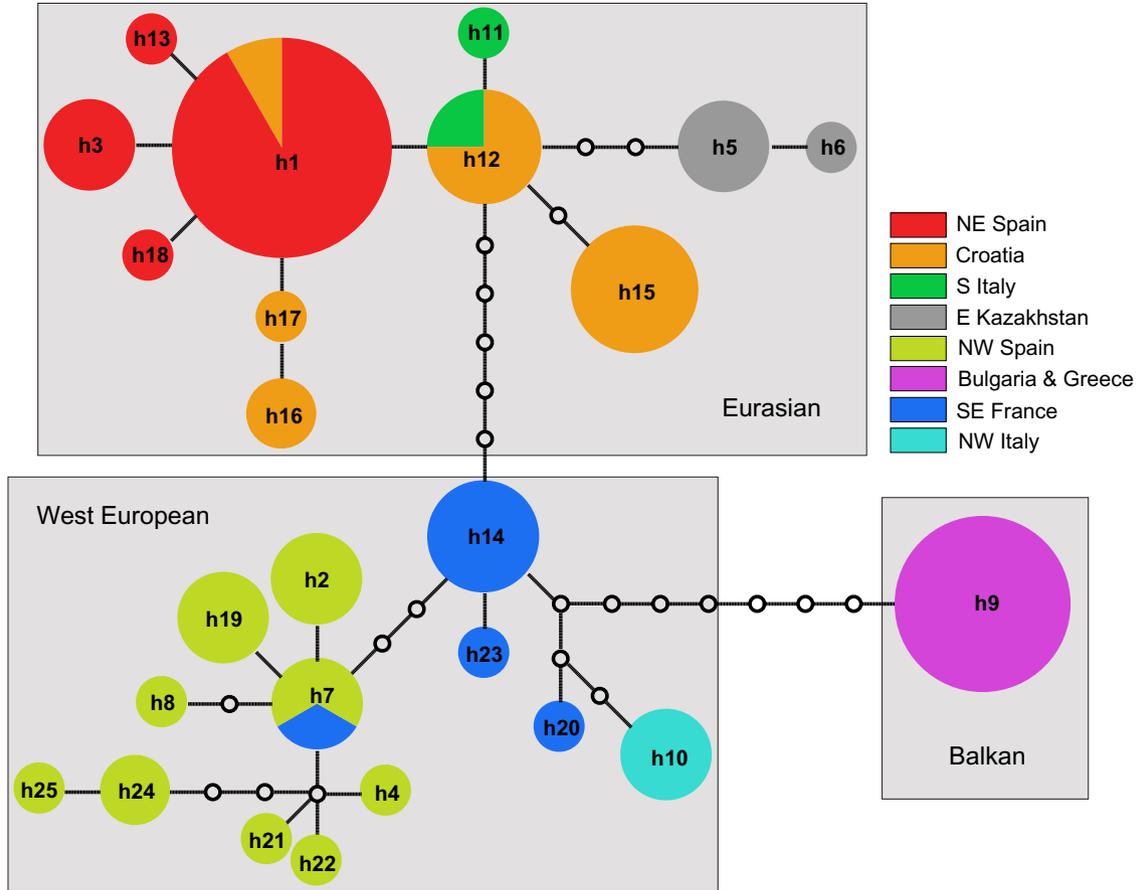


Figure 3. Maximum parsimony haplotype network inferred with a 95% connection limit based on 67 specimens of *Polyommatus ripartii ripartii*. Coloured dots are scaled to represent the relative frequency of each haplotype in the data set. Each branch represents one point mutational step, and the small black circles represent ‘missing’ haplotypes. The grey boxes indicate the three main genetic lineages found in Europe.

Croatian population of *Polyommatus* with *ripartii*-like morphology were recovered within the Eurasian clade of *P. r. ripartii*, which confirms that they belong to this taxon (see Appendix S1 for details on the Croatian population). Within the same Eurasian clade, all the Kazakhstani and Mongolian specimens formed a supported subgroup.

The three main clades of *P. r. ripartii* were also reflected by the haplotype network (Fig. 3). The haplotype diversity appeared to be highest in the West European clade (13 haplotypes, based on 25 specimens), followed by the Eurasian clade (11 haplotypes, based on 40 specimens). The Balkan clade was represented by a single haplotype shared among the eight sequenced specimens.

At a regional scale, it is worth noting the high haplotype diversity of the Spanish specimens belonging to the West European clade (nine haplotypes in 16 specimens), and of the French specimens from the same clade (four haplotypes in seven specimens). By contrast, the Eurasian clade specimens from north-eastern Spain had four haplotypes in 16 specimens.

MORPHOLOGICAL DATA

Valva length varied considerably among individuals, even over relatively small geographic areas

(Tables S2, S3). When plotting valva length against forewing width, no clear differences could be noticed among the four *P. ripartii* genetic lineages recovered by *COI* and included in the morphometric analyses (Eurasian, $n = 18$; West European, $n = 18$; Balkan, $n = 3$; the subspecies *paralcestis*, $n = 2$; Fig. S2). Specimens of the Eurasian clade displayed the highest variability, but this may not be so surprising as they also originate from very distant areas. The two smallest specimens belong to the Eurasian lineage from the Huesca province (Aragón, Spain), in the area where the West European and the Eurasian lineages come into closest vicinity (Fig. 1, Supporting Information, Tables S2, S3).

For 31 specimens, we were also able to calculate the ratio between forewing length and valva length. With one exception, all our *P. ripartii* specimens had an index higher than six (Tables S2, S3). The single male specimens available of *P. nephohiptamenos* and *P. admetus* also had indexes higher than six (Table S2).

In terms of wing pattern, the *P. ripartii* specimens sequenced by us displayed considerable phenotypic variability (Figs S3, S4), mainly regarding characters from the underside of the wings such as the presence, absence, or intensity of the white stripe on the hindwings, the size and number of black spots, and ground colour. Even when only specimens from a restricted geographical area were considered, variability was rather pronounced, and no constant differences were observed among genetic lineages (see Appendix S1 for further details on genitalia and wing morphology).

DISCUSSION

GENERAL PATTERNS IN *POLYOMMATUS RIPARTII* AND RELATED TAXA

Polyommatus ripartii is most likely of Asian origin, and the centre of radiation for the whole subgenus is actually in Asia Minor, the Caucasus, and Iran (Wiemers, 2003; Kandul *et al.*, 2004). It should, however, be noted that both Vila *et al.* (2010) and our study did not recover *P. ripartii* as monophyletic: the main lineages of *ripartii* are well supported, but the monophyly of the species as a whole is unresolved because of unsupported relationships with *P. admetus*, *P. khorasanensis*, and *P. nephohiptamenos* (Fig. 2).

Polyommatus admetus is one of the few species of the 'brown type' that can be readily identified based on external morphology (e.g. Tolman & Lewington, 2008), and its specific status is not questionable. Moreover, it has a haploid chromosome number of $n = 77-80$ (e.g. Lesse, 1960; Lukhtanov & Dantchenko, 2002; Wiemers, 2003; Vila *et al.*, 2010), which is clearly different from that of *P. ripartii*

($n = 90$; e.g. Lesse, 1960, Munguira *et al.*, 1995; Coutsis, Puplesiene & De Prins, 1999; Vila *et al.*, 2010). Although our study is not focused on *P. admetus*, it is worth noting that the specimens analysed form two well-supported lineages that are geographically correlated: a western one including the Balkans and western Turkey, and an eastern one including eastern Turkey, Armenia, and Azerbaijan (Fig. 2; Table S1).

Polyommatus khorasanensis is morphologically similar to *P. ripartii*, but has a haploid chromosome number of $n = 84$ (Lesse, 1963; Lukhtanov *et al.*, 2005). *Polyommatus nephohiptamenos* is morphologically similar to *P. ripartii* (Brown & Coutsis, 1978; Kolev, 1994), and has a haploid chromosome number of $n = 84-(-)88$ (Coutsis & De Prins, 2007). *Polyommatus nephohiptamenos* has been considered as conspecific with *P. ripartii* in some recent publications (Kudrna *et al.*, 2011). Thus, the lack of monophyly for *P. ripartii* in both our data set and in the data set of Vila *et al.* (2010) could be interpreted as resulting from a lack of resolution of the selected markers and/or unresolved taxonomy. As our material for controversial taxa such as *nephohiptamenos* was limited, and a revision of such taxa is beyond the scope of our article, we refrain from drawing taxonomic conclusions and limit the interpretation of the results to *P. ripartii* as defined by Vila *et al.* (2010). Further studies dealing with the taxonomic status of *nephohiptamenos* and *khorasanensis* are certainly needed.

PARTICULARITIES OF THE MAIN EUROPEAN LINEAGES OF *POLYOMMATUS RIPARTII*

Vila *et al.* (2010) provisionally recognized three subspecies of *P. ripartii* (*P. r. ripartii*, *P. r. paralcestis*, and *P. r. colemani*), of which only the first occurs in Europe. Genetic structure was noted within Europe, but the topic was not discussed in detail because of a different focus of the article and sampling limitations. Our data confirm the fact that *P. r. colemani* and *P. r. paralcestis* are well differentiated from the other *P. ripartii* individuals, but a better sampling and more in-depth studies are needed to understand their distribution and taxonomic status. However, additional sampling of *P. ripartii* and novel European populations produced a better picture of the biogeography of the species in Europe. It becomes clear that three main mitochondrial lineages occur in Europe (here named Eurasian, Balkan, and West European).

The Eurasian lineage comprises specimens from a very large area, ranging from Mongolia and eastern Kazakhstan to several European populations. In Europe, this lineage has a highly fragmented distribution with several isolates in European Russia, southern Ukraine (Crimea), Croatia, southern Italy,

and north-eastern Spain. It is worth noting the key geographic position of the newly discovered Croatian *P. ripartii* populations, which is reflected in terms of genetic diversity. Indeed, although a strong genetic structure is the rule, Croatia displays haplotypes also occurring in northern Spain and in southern Italy (Fig. 3), suggesting that a substantial level of haplotype variability has been preserved in this region. The finding of *P. ripartii* in Croatia (Figs 1, S3, S5) fills an important distribution gap in the Mediterranean range of this species, by linking the populations from Western Europe with those from the central and eastern Balkans. Recently, it has been shown that *Polyommatus exuberans* (Susa Valley, north-western Italy), *Polyommatus galloi* (Pollino Mountain, southern Italy), and *Polyommatus agenjoi* (Catalonia, north-eastern Iberia) are actually not species but local populations of *P. ripartii* (Vila *et al.*, 2010). These findings had already suggested that *P. ripartii* may have a more continuous distribution between western and eastern Europe, but a distance of about 1000 km exists between the populations from north-western Italy and those from southern Serbia. The *P. ripartii* populations from Croatia are about 700 km east from those in north-western Italy, and 300 km west from those in Serbia (Kudrna *et al.*, 2011) (see Appendix S1 for more details about the Croatian population).

Another finding worth mentioning is the presence of the Eurasian lineage of *P. ripartii* in the Serranía of Albarracín (Sistema Ibérico), which represent the southernmost known Iberian population of *P. ripartii*. These new findings indicate that the ranges of *P. fabressei* and *P. ripartii* meet in this area: the study of Vila *et al.* (2010) included a specimen of *P. fabressei* from Albarracín that is also used here (Table S1). Until now it has been considered that the Sistema Ibérico is inhabited exclusively by *P. fabressei* (e.g. Munguira *et al.*, 1995; Kudrna, 2002; García-Barros *et al.*, 2004), and only the recent map in Kudrna *et al.* (2011) indicates *P. ripartii* occurring in area. Thus, our data document a part of the contact zone for these parapatric species. However, as *P. fabressei* and *P. ripartii* are extremely similar morphologically (Munguira *et al.*, 1995; Vila *et al.*, 2010), the detailed documentation of their distribution limits will be challenging, and will require DNA sequencing.

The West European lineage displays a disjunct distribution, with isolates in central–northern Spain, in south-eastern France, and in north-western Italy. Rather surprisingly, in north-eastern Spain, between the two main groups of populations of this lineage, a group of populations of the Eurasian lineage occurs (Fig. 1). According to the available data, in the Iberian Peninsula the two lineages come into close vicinity in the north-west of the Huesca province

(Aragón), where they are separated by at most 60 km. The question arises whether the two lineages can be found in sympatry, as there are no obvious geographic barriers. Furthermore, the Huesca province is known to have a large number of *P. ripartii* populations (Munguira *et al.*, 1995; García-Barros *et al.*, 2004). This means that research focused on the region between these two lineages may be rewarding, and, if sympatry is detected, this would allow testing for gene flow.

The Balkan lineage seems to be confined to the Balkan Peninsula (Greece and Bulgaria), and displays a very low haplotype diversity (a single haplotype detected). Further research is needed to clarify whether this lineage penetrates into Turkey or if it can occur in sympatry with the Eurasian lineage. The available data indicate that eastern Turkey is inhabited by a distinct taxon (*P. r. paralcestis*; Figs 1, 2; see Vila *et al.*, 2010), but specimens from western Turkey have not yet been sequenced.

Our analyses indicated that the different genetic lineages of *P. ripartii* cannot be reliably distinguished based on morphology (Figs S2–S4). Furthermore, morphological characters seem to be of only limited use for distinguishing *P. ripartii* from other similar species (see Appendix S1 for further details). It is clear that the genetic differentiation undergone by this species has not been accompanied by changes in morphology. We strongly recommend that not only descriptions of new *Agrodiaetus* taxa, but also reports of new populations (especially if they are isolated), include molecular and/or karyological data unless solid morphological differences are at play. The importance of including molecular-based methods in taxonomical studies is highlighted by several new butterfly species that have been reported in the European fauna in the last few years by combining multiple sources of data (e.g. Nazari & Sperling, 2007 and Dapporto, 2010 for *Zerynthia cassandra*; Dincă *et al.*, 2011 for *Leptidea juvernica*; Dincă, Dapporto & Vila, 2011 for *Polyommatus celina*).

BIOGEOGRAPHY OF *POLYOMMATUS RIPARTII* IN EUROPE

Our dating estimated that the most recent common ancestor of the *P. ripartii* subspecies dates back to the late Pleistocene, c. 0.87 Mya, in accordance with previous findings (Vila *et al.*, 2010). It should, however, be noted that time estimates in the absence of fossil records to calibrate the phylogenetic trees are subject to wide errors. Thus, these dates can only be taken as approximate estimations that provide a rough time frame to the main evolutionary events in the history of the species. The presence of three lineages and their fragmented distribution indicate a complex

colonization history of Europe by this species. As two of these lineages (West European and Balkan) are apparently endemic to Europe, it is likely that they diverged in European glacial refugia (Iberia and the Balkans) during the Pleistocene (Taberlet *et al.*, 1998; Hewitt, 2000). The West European lineage appears to have fragmented into Spanish and French populations about 0.26 Mya, whereas the Balkan lineage expanded very recently between southern Greece and Bulgaria, about 0.08 Mya (Fig. 1). Several studies performed on both vertebrates and invertebrates indicate that the Balkan Peninsula refugium usually allows good recolonization (expansion northwards) because of a lack of strong geographic barriers. The Pyrenees do not seem to be a strong barrier either, whereas expansions from the Italian Peninsula refugium are often blocked by the presence of the Alps acting as a strong barrier (Taberlet *et al.*, 1998; Hewitt, 2000; Schmitt, 2007; Dennis & Schmitt, 2009). Interestingly, the distribution of the three European *P. ripartii* lineages does not seem to fit these patterns as well. It is rather unusual that, although the Iberian and especially the Balkan refugia do not seem to have been able to expand much, the Eurasian lineage that is also present in southern Italy is the most widespread. This suggests that, whereas the Iberian and Balkan peninsulas served as refugia for the West European and Balkan lineages, respectively, it is possible that the Eurasian lineage appeared in central Asia, and subsequently expanded westwards into Europe. Approximately 0.29 Mya it fragmented into several isolated populations, which remain today (Fig. 1). The distribution of these lineages is counterintuitive because of both the rather unusual postglacial expansion patterns and the extremely fragmented distribution of the Eurasian lineage. Furthermore, none of these lineages have been found in sympatry. The genetic structure is most interesting in the Iberian Peninsula, where the West European and Eurasian lineages occur in close proximity (Fig. 1). This pattern suggests the potential presence of mechanisms that prevent their coexistence, and the phenomenon certainly deserves further study. Four non-mutually exclusive potential explanations can be put forward: (1) the small population size of the isolates could easily lead to fixation of the haplotypes, and thus reduce the probability of the long-term existence of multiple lineages in a single locality; (2) the fragmented distribution of the species and the local character of the populations could have prevented contact between different lineages; (3) our sampling was insufficient to reflect the sympatry of different lineages; or (4) some mechanism preventing coexistence is at play. Detailed research in potential lineage contact zones (such as northern Iberia or the Balkans) is desirable to test these hypotheses.

Another interesting aspect is that within the West European lineage, populations from south-eastern France and north-western Italy display different haplotypes (Fig. 3). This suggests that, despite geographical proximity, they are not in contact and have not been for quite some time. Actually, *P. ripartii* populations in these areas are extremely local, especially in north-western Italy. Incidentally, the fact that the valleys in the north-western Italian Alps have long represented an isolated refuge is exemplified by the presence of other unique taxa, such as the Cogne Valley endemic *Polyommatus humedasae* (Toso & Balletto, 1976) (Vila *et al.*, 2010; Kudrna *et al.*, 2011).

The resulting mosaic of scattered populations and lineages across a wide geographical range can only be explained by periods of alternating high dispersal, fragmentation, and strong isolation for the last hundreds of thousands of years. It thus seems that *P. ripartii* is a species strongly affected by changes in climate, and that depending on environmental conditions is able to disperse long distances or, by contrast, remain isolated in small valleys for millennia without genetically mixing with nearby populations. *Polyommatus ripartii* fails to align with the usual biogeographical patterns inferred for European butterflies (Schmitt, 2007; Dennis & Schmitt, 2009). Therefore, this is another example of a series of recent studies (e.g. Valdiosera *et al.*, 2008; Dapporto *et al.*, 2011; Dincă, Dapporto & Vila, 2011) that challenge previous findings, reveal unexpected layers of complexity in biogeography, and underline the need for further large-scale studies on multiple taxa in order to obtain a clearer picture on biogeographical patterns in Europe.

CONSERVATION OF *POLYOMMATUS RIPARTII* IN EUROPE

From a conservation point of view, *P. ripartii* is considered as not threatened in Europe according to the International Union for Conservation of Nature (IUCN) criteria, but has been catalogued as near threatened in the European Union (Van Swaay *et al.*, 2010). Although it is not as localized and potentially endangered as other 'brown type' *Agrodiaetus* taxa (e.g. *humedasae*, *violetae*, and *orphicus*; Van Swaay *et al.*, 2010), our findings indicate that the highly fragmented genetic lineages of *P. ripartii* could be considered as evolutionarily significant units (ESUs) for conservation (Ryder, 1986; Moritz, 1994; Avise, 2000). Two of these ESUs (West European and Balkan) appear to have fairly restricted distributions confined to Europe. Moreover, the complex distribution pattern displayed by the three lineages identified in Europe offers the possibility to study interesting

phenomena such as the potential mutual exclusion between lineages in the Iberian Peninsula.

Polyommatus ripartii is also under moderate risk from a climatic point of view (Settele *et al.*, 2008), with projected losses of its climatic niche, especially in the case of the Balkan lineage, although one should note that these projections do not include the most recent taxonomical and distributional changes for this taxon.

CONCLUSION

Our study revealed the presence of three well-defined genetic lineages within the European populations of *P. ripartii*. These lineages display a complex distribution pattern that does not correspond with the most common biogeographic patterns of European fauna. The distribution of *P. ripartii* seems to have been modelled by several glacial and interglacial periods, resulting in a mosaic of genetic lineages that apparently do not extensively coexist. The causes behind this interesting pattern deserve further attention, and northern Spain, where the West European and Eurasian lineages are likely to meet, offers a great opportunity to test the hypotheses we put forward. We also reported a new population of *P. ripartii* in Croatia, which reduces the distributional gap between the western and eastern populations of the species, while also allowing for a better understanding of its biogeography.

Morphology is shown to be of very limited use when dealing with both intra- and interspecific differentiation, and taxonomic conclusions should not be drawn without molecular and/or karyological data. The case of *P. ripartii* shows that not all species can be easily accommodated within the European biogeographic glacial–postglacial classical models, and underlines the great importance of preserving genetic diversity, a reflection of the history and evolution of the species.

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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.** Elements of the male genitalia and forewings measured and included in the morphometric analysis.
- Figure S2.** Bivariate scatter plot of valva length and forewing width in 41 male specimens of *Polyommatus ripartii* identified through phylogenetic analysis of mitochondrial DNA sequences.
- Figure S3.** Dorsal and ventral view of representative male and female specimens of Croatian *Polyommatus ripartii* included in this study.
- Figure S4.** Dorsal and ventral view of representative male specimens of *Polyommatus ripartii* included in this study.
- Figure S5.** Views of the habitat of *Polyommatus ripartii* on Mosor Mountain, Croatia (photographs by M. Runquist).
- Table S1.** List of specimens used in this study.
- Table S2.** Specimens used for morphometry of the wings and male genitalia.
- Table S3.** Values for measurements of male *Polyommatus ripartii*, *Polyommatus orphicus*, and *Polyommatus aroaniensis* valva length, forewing length, and the forewing length/valva length index.
- Appendix S1.** Supplementary results and discussion for the morphological analyses, and data about *Polyommatus ripartii* in Croatia.