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How common are dot-like distributions? Taxonomical oversplitting in western European *Agrodiaetus* (Lepidoptera: Lycaenidae) revealed by chromosomal and molecular markers

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Approximately 50 taxa of butterflies in Western Europe have been described as new species or elevated to the level of species during the last 40 years. Many, especially those belonging to the genus *Agrodiaetus*, have unusually localized, 'dot-like' distributional ranges. In the present study, we use a combination of chromosomal and molecular markers to re-evaluate the species status of *Agrodiaetus* distributed west of the 17th meridian. The results obtained do not support the current designations of *Agrodiaetus galloi*, *Agrodiaetus exuberans*, and *Agrodiaetus agenjoi* as endemic species with highly restricted distribution ranges, but indicate that these taxa are more likely to be local populations of a widely distributed species, *Agrodiaetus ripartii*. *Agrodiaetus violetae* is shown to be a polytypic species consisting of at least two subspecies, including *Agrodiaetus violetae subbaeticus* **comb. nov.** and *Agrodiaetus violetae violetae*. *Agrodiaetus violetae* is genetically (but not chromosomally) distinct from *Agrodiaetus fabressei* and has a wider distribution in southern Spain than previously believed. *Agrodiaetus humedasae* from northern Italy is supported as a highly localized species that is distinct from its nearest relatives. We propose a revision of the species lists for *Agrodiaetus* taking these new data into account. The results reported in the present study are relevant to animal conservation efforts in Europe because of their implications for IUCN Red List priorities.

ADDITIONAL KEYWORDS: systematics – species – conservation – distribution – range – reinforcement – phylogeny.

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INTRODUCTION

Comparison of the first comprehensive work on European butterflies (Higgins & Riley, 1970) with more recent publications (de Prins & Iversen, 1996; Tolman, 1997; Kudrna, 2002; Lafranchis, 2004; Dennis *et al.*, 2008) shows that approximately 50 butterfly taxa have been described as new species or elevated to species rank during the last 40 years. Many of these newly-recognized species have extremely local 'dot-like' distributions that are restricted to particular mountain valleys in Spain, Italy, the Balkan Peninsula and Crimea, or to small Mediterranean islands (Kudrna, 2002). Usually, these dot-like distributed taxa are geographically isolated populations whose morphological and ecological differences from their closest relatives have rarely been assessed. In theory, species with such restricted ranges may represent either relicts of species that had much broader distributions in the past, or young species that originated recently and have not yet expanded their ranges. However, before considering these possibilities, a more thorough consideration must be made of whether these nominal taxa are indeed valid species rather than isolated populations of other known species with broader distributions.

Species in the butterfly genera and subgenera *Agrodiaetus*, *Hipparchia*, *Plebejus*, *Lysandra*, and *Polyommatus* make up a large proportion of those with dot-like distributions. These groups are among the most species-rich genera of European butterflies, and a number include taxa in the process of speciation. The genus *Agrodiaetus* (considered by some to be a subgenus of the large genus *Polyommatus*) is especially interesting in this respect. *Agrodiaetus* comprises a taxonomically diverse group of blue butterflies (Forster, 1956–1961; Eckweiler & Häuser, 1997; Wiemers, 2003; Kandul *et al.*, 2004; Wiemers, Keller & Wolf, 2009). The monophyly of the genus is strongly supported by molecular data (Kandul *et al.*, 2002, 2004; Wiemers, 2003; Wiemers *et al.*, 2009). Adults of *Agrodiaetus* have a wingspan of only 2–4 cm, and the sexes are often dimorphic, with females typically brown and males blue on the upper surface of their wings. This blue coloration is plesiomorphic, and is found in many species in closely-related genera of the *Polyommatus* section (Kandul *et al.*, 2004). Phylogenetic evidence suggests that reinforcement of pre-zygotic reproductive isolation is likely to have given rise to different male wing coloration in this group: males can have brown, white, silver, violet, and even orange wings, and quite a few of those with light wing coloration also reflect ultraviolet light (Lukhtanov *et al.*, 2005). Given that the number of species of *Agrodiaetus* (at least 120) is much greater than the variety of colours displayed by males, and other diagnostic morphological characters are scarce, the genus may also include cryptic species.

The most remarkable characteristic of the genus *Agrodiaetus* is its unusual diversity of chromosomal complements, or karyotypes. Species of *Agrodiaetus* exhibit among the highest range in chromosome number in the animal kingdom. The karyotype is generally stable within species, although differences between closely-related species are often high. Haploid chromosome numbers in *Agrodiaetus* range from $n = 10$ in *Agrodiaetus caeruleus* to $n = 134$ in *Agrodiaetus shahrami* (Lukhtanov & Dantchenko, 2002a; Lukhtanov *et al.*, 2005).

Modern lists of European *Agrodiaetus* include 13–22 species, depending on the taxonomic interpretation of species or subspecies status for a number of taxa (De Prins & Iversen, 1996; Dennis, 1997; Kudrna, 2002; Dennis *et al.*, 2008). Some of these taxa have quite broad distributions. However, eleven species of European *Agrodiaetus* (i.e. approximately one-half the current species list) have been described to have dot-like distributions and to be restricted to particular mountains or valleys in Spain, Italy, the Balkan Peninsula, and Crimea. These are: (1) *Agrodiaetus violetae* (southern Spain: Sierra de la Almijara); (2) *Agrodiaetus fulgens* (north-eastern Spain: Catalonia); (3) *Agrodiaetus agenjoi* (north-eastern Spain: Catalonia); (4) *Agrodiaetus exuberans* (north-western Italy: Susa); (5) *Agrodiaetus humedasae* (north-western Italy: Cogne Valley); (6) *Agrodiaetus galloi* (southern Italy: Calabria); (7) *Agrodiaetus nephohiptamenos* (southern Bulgaria and northern Greece: Pirin, Orvilos, Pangeon and Phalakron Mountains); (8) *Agrodiaetus eleniae* (northern Greece: Mount Phalakron); (9) *Agrodiaetus orphicus* (southern Bulgaria and northern Greece: Mount Rhodope); (10) *Agrodiaetus budashkini* (Ukraine: Crimea); and (11) *Agrodiaetus pljushtchi* (Ukraine: Crimea). We analyzed three of these nominal species (*A. budashkini*, *A. pljushtchi*, and *A. fulgens*) in previous studies (Kandul *et al.*, 2004; Lukhtanov, Vila & Kandul, 2006; Lukhtanov & Budashkin, 2007). The present study addresses the status of *A. violetae*, *A. agenjoi*, *A. exuberans*, *A. humedasae*, *A. galloi* and related taxa from south-west Europe, and includes a general analysis of the problem of dot-like species ranges in *Agrodiaetus*.

All these target taxa have brown wing coloration in both males and females, and are difficult to distinguish using traditional morphological characters. The first step to characterize such species typically involves molecular methods. However, the use of standard molecular markers such as short fragments of the mitochondrial gene COI and the noncoding nuclear sequence, internal transcribed spacer 2 (ITS2), is sometimes insufficient to distinguish

between evolutionarily young sister species, either because they may be weakly differentiated with respect to these markers (Wiemers, 2003; Kandul *et al.*, 2004; Wiemers & Fiedler, 2007; Lukhtanov *et al.*, 2009) or because they are too polymorphic (Lukhtanov & Shapoval, 2008; Lukhtanov, Shapoval & Dantchenko, 2008). An absence of lineage sorting among species can be frequently a problem for the use of molecular markers in rapidly evolving taxa of *Agrodiaetus*: the time to coalescence for alleles within lineages may be greater than the time subsequent to speciation (Kandul *et al.*, 2004).

Chromosomal characters in many groups may evolve more quickly, and because they are often present as fixed differences, can sometimes provide better markers for recently evolved taxa (King, 1993; Dobigny *et al.*, 2005). The study of the karyotype provides good diagnostic characters for most *Agrodiaetus* species and, as such, has become an important requirement for describing and delimiting new taxa (de Lesse, 1960a; Lukhtanov & Dantchenko, 2002b; Lukhtanov *et al.*, 2003, 2006). As with molecular data, cytological data have their own limitations; they may be incapable of resolving groups of species characterized by extreme chromosomal conservatism. However, molecular and chromosomal approaches are complementary, and applying a combination of these approaches can provide powerful taxonomic insights, especially when considered with morphological and ecological data (Lukhtanov *et al.*, 2006; Descimon & Mallet, 2009).

Dot-like distributed species present practical as well as theoretical difficulties. Increasing the number of such species substantially increases the potential conservation load for European butterflies (Dennis, 1997). Endemic species, those with small or restricted ranges, are in greater danger of becoming extinct through systematic or stochastic changes in the environment than are widely distributed species (Gaston, 1994). Thus, even if restricted range is not the only factor taken into account, it is not surprising that several local European *Agrodiaetus* taxa are listed among species of conservation concern (Van Swaay *et al.*, 2010).

SPECIES AND SUBSPECIES CONCEPTS

SPECIES

In the present study, we adopt a classification based on the biological species concept (BSC) (Poulton, 1904; Mayr, 1963; Häuser, 1987). Under the BSC, actual or potentially reproductively isolated entities are classified as species. Isolation may not necessarily be complete, but it should be strong enough to prevent taxa from merging when they occur in sympatry (Mayr, 1963; Coyne & Orr, 2004). In practice, the existence of isolation can be tested most effectively via the genotypic cluster approach (Mallet, 2001, 2006; Mallet & Willmott, 2003), in which data on morphological, genetic, ecological, and behavioural characters in a local area are used as evidence of distinctness in sympatry. Genotypic clusters in sympatry can be seen in phenotypic data as a bimodal distribution of traits, and in genetic data as a deficit of heterozygotes or as the presence of linkage disequilibrium among genes. Species recognition through linkage disequilibrium analysis of unlinked genetic markers has already been used in *Agrodiaetus* (Lukhtanov & Shapoval, 2008).

However, when taxa are allopatric, the direct application of the BSC may be more difficult. We suggest that allopatric taxa be considered species if they are clearly distinct with respect to characters that contribute to pre- or post-zygotic reproductive isolation. In the case of *Agrodiaetus*, a strong difference in the colour of the upper side of the male wing (e.g. blue versus brown) most likely contributes to pre-zygotic isolation (Lukhtanov *et al.*, 2005).

Chromosome differences can also be considered indirect evidence for reproductive isolation between taxa in allopatry. It is well known that chromosome rearrangements can cause sterility (King, 1993), and even relatively small differences in chromosome structure can result in post-zygotic isolation (Ferree & Barbash, 2009). However, this is not always true and, in some cases, heterozygosity for chromosome rearrangements does not result in sterility (Nagaraju & Jolly, 1986). Indeed, there is no well-established general rule to determine how many or what types of chromosome rearrangements can be tolerated before resulting in infertile offspring.

The chromosome number of *Agrodiaetus* is generally stable within populations of this genus and, in only a few cases, a limited amount of variability in intra-population haploid chromosome number has been observed. The range of this variation has never exceeded four chromosomes, which we infer is the likely upper threshold of chromosome number differences compatible with offspring fertility in this group (Lukhtanov & Dantchenko, 2002b; Lukhtanov, Wiemers & Meusemann, 2003). Thus, empirical observations of *Agrodiaetus* suggest that a fixed difference of five or more chromosomes in haploid number sets (which is equal to ten or more chromosomes in diploid number sets) provides a useful criterion to use in designating allopatric chromosome races as nonconspecific until direct evidence for the presence/absence of reproductive isolation can be obtained.

Molecular data alone, even in the case of a relatively high level of genetic differentiation between the taxa under comparison, are not sufficient to define biological species because the divergence of standard genetic markers between distinct sympatric species can be low or absent, and intraspecific variation can be relatively high (Lukhtanov *et al.*, 2009). However, genetic divergence comparisons may be useful in highlighting potentially interesting monophyletic lineages that deserve further study, and in identifying morphologically similar species that are not closely related. For example, in the present study, the brown-coloured *Agrodiaetus fabressei* is not sister to the morphologically similar *A. violetae*, but to two blue-coloured species, *Agrodiaetus dolus* and *A. fulgens*. Both mitochondrial and nuclear markers support this result, and thus we consider *A. fabressei* and *A. violetae* not to be conspecific.

Subspecies

Diagnosable allopatric entities (populations or groups of populations) with fixed difference(s) in morphological and/or chromosomal characters should be classified as subspecies if they do not correspond to the species criteria specified above. In general, we agree with Descimon & Mallet (2009), that 'there is justification for reviving the rather neglected (and misused) rank of subspecies, with the trend among lepidopterists to consider only more strongly distinct forms (in morphology, ecology, or genetics) as subspecies, and to lump dubious geographic forms as synonyms . . . [This provides] . . . a useful compromise between descriptions of geographic variation, the needs of modern butterfly taxonomy, and Darwin's pragmatic use of the term species in evolutionary studies.'

MATERIAL AND METHODS

Taxon sampling

In the present study, we focus only on those taxa found in Europe west of the 17th meridian. In this region, almost all *Agrodiaetus* taxa and populations are concentrated on the Iberian Peninsula in France and Italy. Except for *Agrodiaetus damon*, they belong to two groups of species: the *Agrodiaetus admetus* group, and the *A. dolus* group, which are sister clades in all published phylogenetic reconstructions (Wiemers, 2003; Kandul *et al.*, 2004, 2007; Lukhtanov *et al.*, 2005). These two groups also include taxa from the Balkan Peninsula, eastern Europe, and western Asia that are not considered in detail in the present study. However, to estimate relationships among western European taxa, we include in our analysis all eastern European and non-European species except the Anatolian–Iranian species, *Agrodiaetus demavendi*, where specimens with unambiguous species determination and precise chromosome number count were not available (Tables 1, 2).

When collecting in the field, we used a protocol that allowed us to obtain molecular and chromosomal information from the same individual specimens (Bulatova *et al.*, 2009). Additionally, we tried to obtain samples from the type localities of each studied taxa in order to connect the chromosomal and molecular data with correct species names. In particular, *A. violetae*, *Agrodiaetus fabressei subbaeticus*, *A. exuberans*, *Agrodiaetus ripartii susae*, *A. humedasae* and *A. galloi* were collected from their type-localities. Specimens RV-03-H463 and RVcoll. 07-F038 of *A. agenjoi* were collected approximately 6.5 km and 125 km, respectively, from the taxon type locality, 'Barcelona, Taradell' [Barcelona province, Catalonia, north-east Spain] (Forster, 1965). Specimens RE-07-G266 and RE-07-G273 of *Agrodiaetus ripartii rippertii* were collected approximately 100 km north-west from the taxon type locality, 'aux environs de Digne' [Alpes de Haute Provence, France]) (Boisduval, 1832).

We also inspected the morphology and taxon identification of samples whose sequences we downloaded from GenBank. In doing so, we found that samples MW01105 and MAT-99-Q878 from Catalonia, previously identified as *A. ripartii* (Wiemers, 2003; Kandul *et al.*, 2004), have no white streak on the underside of the hind wing. Although this character can be labile, if we take it into account in conjunction with the collecting locality, we consider that these specimens actually belong to the nominal species, *A. agenjoi*.

Karyotyping

Only fresh adult males were used for karyotyping. Adults were collected in the field, and after they were killed by a sharp pinch to the thorax, testes were immediately excised and placed into 0.5-mL vials with freshly prepared Carnoy fixative (ethanol and glacial acetic acid, 3 : 1). Bodies were preserved in 2-mL plastic vials with 100% ethanol for DNA analysis, and wings were stored in glassine envelopes.

Gonads were stored in fixative for 2–6 months at 4 °C and then stained with 2% acetic orcein for 30 days at 20 °C. Cytogenetic analysis was conducted using a two-phase method of chromosome analysis (Lukhtanov & Dantchenko, 2002a; Lukhtanov *et al.*, 2006). Chromosome preparations are stored in the Department of Entomology of St Petersburg State University, Russia. Butterfly bodies in ethanol, and wings in glassine envelopes are stored in the Lepidoptera DNA and Tissues Collection at the Museum

Table 1. List of the *Agrodiaetus* samples used in the present study

(Traditionally) accepted name and combination	Proposed name and combination	Sample code	Locality
Agrodiaetus admetus	*Agrodiaetus admetus*	AD-00-P016	Armenia, Aiodzor Mts, Gnishyk
Agrodiaetus admetus	*Agrodiaetus admetus*	JC 01014	Greece, Peloponnisos, Mt Taiyetos, 1200–1300 m
Agrodiaetus admetus	*Agrodiaetus admetus*	MW98084	Turkey, Antalya, Cukurelma N Elmali 1300 m
Agrodiaetus admetus anatoliensis	*Agrodiaetus admetus anatoliensis*	VL-01-L101	Turkey, Gümüshane, Torul
Agrodiaetus admetus malievi	*Agrodiaetus admetus malievi*	VL-03-F903	Azerbaijan, Talysh, Zuvand
Agrodiaetus agenjoi	*Agrodiaetus ripartii ripartii*	MAT-99-Q878	Spain, Lleida, Tremp, Rúbies
Agrodiaetus agenjoi	*Agrodiaetus ripartii ripartii*	MW01105	Spain, Tarragona, Santa Coloma de Queralt, 700 m
Agrodiaetus agenjoi	*Agrodiaetus ripartii ripartii*	RV-03-H463	Spain, Barcelona, El Brull, 830 m
Agrodiaetus agenjoi	*Agrodiaetus ripartii ripartii*	RVcoll.07-F038	Spain, Tarragona, Serra de Prades, Barranc de Vinarroig, 920 m
Agrodiaetus ainsae	*Agrodiaetus fulgens ainsae*	MAT-99-Q894	Spain, Lleida, Tremp, Rúbies
Agrodiaetus ainsae	*Agrodiaetus fulgens ainsae*	MW01001	Spain, Álava, Ilarduya, W Eguino, 550 m
Agrodiaetus ainsae	*Agrodiaetus fulgens ainsae*	MW01053	Spain, Huesca, Embalse de la Peña, Sta. María, 500 m
Agrodiaetus ainsae	*Agrodiaetus fulgens ainsae*	MW01078	Spain, Huesca, Embalse de la Peña, Triste, 600 m
Agrodiaetus alcestis	*Agrodiaetus alcestis*	MW98212	Turkey, Adana, Saimbeyli, 1500 m
Agrodiaetus alcestis	*Agrodiaetus alcestis*	MW98315	Turkey, Karaman, Ermenek, Yellibeli Geçidi, 1800 m
Agrodiaetus alcestis karacetinae	*Agrodiaetus alcestis karacetinae*	MW00229	Iran, Zanjan, Qazayd Dagh, 25 km O. Zanjan, 2300 m
Agrodiaetus alcestis karacetinae	*Agrodiaetus alcestis karacetinae*	MW00231	Iran, Zanjan, Qazayd Dagh, 25 km O. Zanjan, 2300 m
Agrodiaetus alcestis karacetinae	*Agrodiaetus alcestis karacetinae*	MW99380	Turkey, Hakkari, 22 km NW Yüksekova, 1800 m
Agrodiaetus alcestis karacetinae	*Agrodiaetus alcestis karacetinae*	VL-03-F669	Iran, Markazi, Khiru
Agrodiaetus aroaniensis	*Agrodiaetus aroaniensis*	JC00040	Greece, Peloponnisos, Mt Helmos, 1350 m
Agrodiaetus damocles krymaeus	*Agrodiaetus damocles krymaeus*	NK-00-P103	Ukraine, Crimea, Kurortnoe
Agrodiaetus damon	*Agrodiaetus damon*	MAT-99-Q841	Spain, Girona, Pyrenees Mts, Urús
Agrodiaetus dantchenkoi	*Agrodiaetus dantchenkoi*	MW99274	Turkey, Van, Gürpinar, Kurubas Geçidi, 2200 m
Agrodiaetus dantchenkoi	*Agrodiaetus dantchenkoi*	MW99276	Turkey, Van, Gürpinar, Kurubas Geçidi, 2200 m
Agrodiaetus dantchenkoi	*Agrodiaetus dantchenkoi*	MW99319	Turkey, Van, 25–32 km N Çatak, 2000–2200 m
Agrodiaetus dantchenkoi	*Agrodiaetus dantchenkoi*	MW99320	Turkey, Van, 25–32 km N Çatak, 2000–2200 m
Agrodiaetus dantchenkoi	*Agrodiaetus dantchenkoi*	VL-01-L342	Turkey, Van, Çatak
Agrodiaetus dolus virgilia	*Agrodiaetus dolus virgilia*	RE-07-G106	Italy, Rocca Pia, 1215 m
Agrodiaetus dolus vittatus	*Agrodiaetus dolus vittatus*	MAT-99-Q923	France, Languedoc Reg, Mende
Agrodiaetus eriwanensis	*Agrodiaetus eriwanensis*	AD-00-P303	Armenia, Aiodzor Mts, Gnishyk
Agrodiaetus erschoffii	*Agrodiaetus erschoffii*	AD-02-L274	Iran, Gorgan, Shahkuh
Agrodiaetus exuberans	*Agrodiaetus ripartii ripartii*	RE-07-G229	Italy, Susa Valley, Urbiano, Mompantero, 720 m
Agrodiaetus fabressei fabressei	*Agrodiaetus fabressei fabressei*	JM00001	Spain, Cuenca, Tragacete, Mogorrita
Agrodiaetus fabressei fabressei	*Agrodiaetus fabressei fabressei*	MAT-99-Q972	Spain, Cuenca, Una, 970 m
Agrodiaetus fabressei fabressei	*Agrodiaetus fabressei fabressei*	MAT-99-Q984	Spain, Albarracín, Puerto de la Losilla
Agrodiaetus fabressei fabressei	*Agrodiaetus fabressei fabressei*	MW01039	Spain, Soria, Sierra de Cabrejas, Abejar, 1100 m
Agrodiaetus fabressei fabressei	*Agrodiaetus fabressei fabressei*	RV-03-H596	Spain, Castelló, Coll d'Ares, 1148 m
Agrodiaetus fabressei subbaeticus	*Agrodiaetus violetae subbaeticus*	RV-03-H554	Spain, Granada, Sierra de la Sagra, 1775 m
Agrodiaetus fabressei subbaeticus	*Agrodiaetus violetae subbaeticus*	RV-03-H555	Spain, Granada, Sierra de la Sagra, 1775 m
Agrodiaetus fabressei subbaeticus	*Agrodiaetus violetae subbaeticus*	RV-03-H556	Spain, Granada, Sierra de la Sagra, 1702 m
Agrodiaetus fabressei subbaeticus	*Agrodiaetus violetae subbaeticus*	RV-03-H557	Spain, Granada, Sierra de la Sagra, 1702 m
Agrodiaetus fabressei subbaeticus	*Agrodiaetus violetae subbaeticus*	RV-03-H558	Spain, Granada, Sierra de la Sagra, 1702 m
Agrodiaetus fabressei subbaeticus	*Agrodiaetus violetae subbaeticus*	RV-03-H560	Spain, Granada, Sierra de la Sagra, 1702 m
Agrodiaetus fulgens	*Agrodiaetus fulgens fulgens*	MAT-99-Q910	Spain, Tarragona, Santa Coloma de Queralt
Agrodiaetus fulgens	*Agrodiaetus fulgens fulgens*	MW01107	Spain, Tarragona, Santa Coloma de Queralt, 700 m
Agrodiaetus galloi	*Agrodiaetus ripartii ripartii*	RE-07-G436	Italy, Calabria, Serra del Prete, Mont Pollino, 1650 m
Agrodiaetus galloi	*Agrodiaetus ripartii ripartii*	RE-07-G437	Italy, Calabria, Serra del Prete, Mont Pollino, 1650 m
Agrodiaetus galloi	*Agrodiaetus ripartii ripartii*	RE-07-G441	Italy, Calabria, Serra del Prete, Mont Pollino, 1650 m
Agrodiaetus galloi	*Agrodiaetus ripartii ripartii*	RE-07-G445	Italy, Calabria, Serra del Prete, Mont Pollino, 1650 m
Agrodiaetus galloi	*Agrodiaetus ripartii ripartii*	RE-07-G447	Italy, Calabria, Serra del Prete, Mont Pollino, 1650 m
Agrodiaetus humedasae	*Agrodiaetus humedasae*	MW99591	Italy, Aosta, Val di Cogne, Pondel, 900 m
Agrodiaetus humedasae	*Agrodiaetus humedasae*	MW99605	Italy, Aosta, Val di Cogne, Pondel, 900 m
Agrodiaetus humedasae	*Agrodiaetus humedasae*	RE-07-G191	Italy, Aosta, Val di Cogne, Ozien-Visyes, 1000 m
Agrodiaetus humedasae	*Agrodiaetus humedasae*	RE-07-G192	Italy, Cogne Valley, Ozien-Visyes, 1000 m
Agrodiaetus humedasae	*Agrodiaetus humedasae*	RE-07-G193	Italy, Cogne Valley, Ozien-Visyes, 1000 m
Agrodiaetus humedasae	*Agrodiaetus humedasae*	RE-07-G194	Italy, Cogne Valley, Ozien-Visyes, 1000 m
Agrodiaetus humedasae	*Agrodiaetus humedasae*	RE-07-G203	Italy, Aosta, Val di Cogne, Ozien-Visyes, 1000 m
Agrodiaetus interjectus	*Agrodiaetus interjectus*	MW99164	Turkey, Erzurum, 5 km NE. Çiftlik, 1900 m
Agrodiaetus khorasanensis	*Agrodiaetus khorasanensis*	VL-03-F526	Iran, Khorasan, Kopetdagh Mts
Agrodiaetus khorasanensis	*Agrodiaetus khorasanensis*	WE02431	Iran, Khorasan, 5 km SW Firizi, 1700–1900 m
Agrodiaetus menalcas	*Agrodiaetus menalcas*	MW98020	Turkey, Fethiye, Gülübeli Geçidi, W. Elmali, 1500 m
Agrodiaetus menalcas	*Agrodiaetus menalcas*	MW98172	Turkey, Sivas, Gökpinar, Gürün, 1700 m
Agrodiaetus menalcas	*Agrodiaetus menalcas*	MW99494	Turkey, Van, Erek Dagi, 2200 m
Agrodiaetus menalcas	*Agrodiaetus menalcas*	VL-01-L122	Turkey, Dilekyolu, Gümüshane
Agrodiaetus ripartii	*Agrodiaetus ripartii ripartii*	AD-00-P033	Russia, Tula Reg, Tatinki

Table 1. *Continued*

(Traditionally) accepted name and combination	Proposed name and combination	Sample code	Locality
Agrodiaetus ripartii pelopi	*Agrodiaetus ripartii ripartii*	JC00043	Greece, Peloponnisos, Mt Helmos, 1350–1500 m
Agrodiaetus ripartii budashkini	*Agrodiaetus ripartii ripartii*	NK-00-P859	Ukraine, Crimea, Karabi yaila
Agrodiaetus ripartii colemani	*Agrodiaetus ripartii colemani*	NK-00-P822	Kazakhstan, West Tian-Shan
Agrodiaetus ripartii paralcestis	*Agrodiaetus ripartii paralcestis*	MW99068	Turkey, Artvin, Kiliçkaya, Yusufeli, 1350 m
Agrodiaetus ripartii paralcestis	*Agrodiaetus ripartii paralcestis*	MW99196	Turkey, Erzincan, 5 km SE Çaglayan, 1500 m
Agrodiaetus ripartii paralcestis	*Agrodiaetus ripartii paralcestis*	MW99263	Turkey, Van, Kurubas Geçidi, Gürpinar, 2200 m
Agrodiaetus ripartii paralcestis	*Agrodiaetus ripartii paralcestis*	MW99264	Turkey, Van, Kurubas Geçidi, Gürpinar, 2200 m
Agrodiaetus ripartii paralcestis	*Agrodiaetus ripartii paralcestis*	AD-00-P337	Armenia, Pambak Mts, Dzhur-dzhur Pass
Agrodiaetus ripartii paralcestis	*Agrodiaetus ripartii paralcestis*	VL-01-L103	Turkey, Gümüshane
Agrodiaetus ripartii paralcestis	*Agrodiaetus ripartii paralcestis*	VL-01-L166	Turkey, Gümüshane, Dilekyolu
Agrodiaetus ripartii ripartii	*Agrodiaetus ripartii ripartii*	MW01014	Spain, Burgos, Ubierna, 20 km N Burgos, 900 m
Agrodiaetus ripartii ripartii	*Agrodiaetus ripartii ripartii*	MW01072	Spain, Huesca, Triste, Embalse de la Pena, 600 m
Agrodiaetus ripartii rippertii	*Agrodiaetus ripartii ripartii*	RE-07-G266	France, Drôme, Col de la Chaudière, 1025 m
Agrodiaetus ripartii rippertii	*Agrodiaetus ripartii ripartii*	RE-07-G273	France, Drôme, Col de la Chaudière, 1025 m
Agrodiaetus ripartii sarkani	*Agrodiaetus ripartii ripartii*	NK-00-P829	Kazakhstan, Dzhungarian, Alatau Mts, Kolbai
Agrodiaetus ripartii sarkani	*Agrodiaetus ripartii ripartii*	NK-00-P848	Kazahkstan, Tarbagatai Mts, Taskeskan
Agrodiaetus ripartii susae	*Agrodiaetus ripartii ripartii*	RE-07-G254	Italy, Torino, Novalesa-Moncenisio, 1155 m
Agrodiaetus ripartii susae	*Agrodiaetus ripartii ripartii*	RE-07-G255	Italy, Torino, Novalesa-Moncenisio, 1155 m
Agrodiaetus rjabovi	*Agrodiaetus rjabovi*	VL-02-X474	Iran, Gilan, Masuleh
Agrodiaetus rjabovi	*Agrodiaetus rjabovi*	VL-03-F816	Azerbaijan, Talysh, Zuvand
Agrodiaetus surakovi	*Agrodiaetus surakovi*	AD-00-P006	Armenia, Aiodzor Mts, Gnishyk
Agrodiaetus urmiaensis	*Agrodiaetus urmiaensis*	VL-04-E365	Iran, Azarbayjan-e-Gharbi
Agrodiaetus valiabadi	*Agrodiaetus valiabadi*	MW00064	Iran, Mazandaran, Pul-e Zanguleh, 15 km NE Kendevan, 2400 m
Agrodiaetus valiabadi	*Agrodiaetus valiabadi*	MW00498	Iran, Mazandaran, 5 km S. Valiabad, 1900 m
Agrodiaetus violetae	*Agrodiaetus violetae violetae*	FGT-05-J629	Spain, Granada, Sierra de la Almijara
Agrodiaetus violetae	*Agrodiaetus violetae violetae*	FGT-05-J630	Spain, Granada, Sierra de la Almijara
Agrodiaetus violetae	*Agrodiaetus violetae violetae*	RVcoll.08-H299	Spain, Andalucía

of Comparative Zoology, Harvard University, and R. Vila's DNA and Tissues Collection at the Universitat Autònoma de Barcelona.

DNA EXTRACTION AND SEQUENCING

Total genomic DNA was extracted using the DNeasyTM Tissue Kit (Qiagen Inc.) in accordance with the manufacturer's instructions. Published primers were used to amplify mitochondrial cytochrome oxidase subunit *I* (COI), leucine transfer RNA (leu-tRNA), cytochrome oxidase subunit *II* (COII) (Folmer *et al.*, 1994; Simon *et al.*, 1994; Monteiro & Pierce, 2001), and nuclear ITS2 (White *et al.*, 1990). The polymerase chain reaction (PCR) was carried out in 25-mL reactions using a DNA Engine thermal cycler (MJ Research Inc.), and typically contained 0.5 mM of each primer, 0.8 mM dNTPs, 1 ¥ Qiagen PCR buffer with additional $MgCl_2$ to a final concentration of 2 mM and 1.25 units Qiagen Taq DNA polymerase. All reactions were initially denatured at 94 °C for 2 min, and then subjected to 35 cycles of 60 s at 94 °C denaturation, 60 s at 45 °C–56 °C (annealing temperature depended on gene amplified), and 90 s at 72 °C extension. After amplification, double-stranded DNA was purified using QIAquick PCR purification kits (Qiagen).

Primers used for amplification served as sequencing primers. All samples were sequenced in both directions. Cycle sequencing reactions were performed in 12-mL reactions: 1.5 mL of ABI Prism BigDye, version 3.1 (Applied Biosystems Inc.), 1.0 mL of 5 ¥ buffer (buffer: 400 mM Tris at pH 9.0 and 10 mM $MgCl_2$), and 0.33 mL each (10 mM) of primer. The remainder of the mixture was composed of ultra pure water 50–90 ng of template DNA in each reaction. Cycle sequence reaction started with a denaturing step of 94 °C for 2 min, followed by 25 cycles of 10 s at 94 °C, 5 s at annealing temperature, which varied for different gene regions, and 4 min at 60 °C. Sequencing was conducted in a 3100 Genetic Analyzer (Applied Biosystems/Hitachi). Sequences obtained specifically for this study were deposited in GenBank under accession numbers HM210162 to HM210202.

PHYLOGENETIC ANALYSIS

For phylogenetic analysis, we used sequences of COI, leu-tRNA, COII and ITS2 original to the present study, as well as sequences obtained from GenBank that had been included in Kandul *et al.* (2004) and Wiemers & Fiedler (2007) (Table 1). We re-edited some of the sequences from previous studies, and a

Table 2. Data used for karyotype and molecular phylogenetic analyses. GenBank codes for sequences obtained specifically for this study, re-edited or with a new fragment sequenced, are highlighted in bold

Taxon (Traditionally) accepted name and combination)	Sample code	Karyotype analysis	Molecular 45-taxa dataset	Molecular 80-taxa dataset	COI genbank code	COII genbank code	ITS2 genebank code
A. admetus	AD-00-P016		X	X	**AY496711** (re-edited)	**AY496711** (re-edited)	
A. admetus	**JC** 01014	[a]n = 80		X	AY556867		AY556733
A. admetus	MW98084			X	AY556986		
A. admetus anatoliensis	VL-01-L101	[b]n = *ca*80	X	X	AY496710	AY496710	
A. admetus malievi	VL-03-F903	[c]n = 79	X	X	EF104617	EF104617	**HM210176**
A. agenjoi	MAT-99-Q878		X	X	AY496780	AY496780	
A. agenjoi	MW01105			X	AY556962		
A. agenjoi	RV-03-H463		X	X	**EF104603** (re-edited)	**EF104603** (re-edited)	
A. agenjoi	RV-07-F038	[d]n = 90					
A. ainsae	MAT-99-Q894	[k]n = 108-110	X	X	**AY496712** (new part seq)	AY496712	**HM210177**
A. ainsae	MW01001	[k]n = 108-110		X	AY556941		AY556601
A. ainsae	MW01053	[k]n = 108-110		X	AY556954		AY556610
A. ainsae	MW01078	[k]n = 108-110		X	AY556958		
A. alcestis	MW98315	[e]n = 20		X	AY557024		AY556653
A. alcestis	MW98212	[e]n = 21		X	AY557008		AY556641
A. alcestis karacetinae	MW00229	[e]n = *ca*19		X	AY556906		
A. alcestis karacetinae	MW00231	[e]n = *ca*19		X	AY556907		AY556574
A. alcestis karacetinae	MW99380	[e]n = 19		X	AY557090		
A. alcestis karacetinae	VL-03-F669	[b]n = 19	X	X	AY954018	AY954018	
A. aroaniensis	**JC**00040	[f]n = 48		X	AY556856		AY556725
A. damocles krymaeus	NK-00-P103	[g]n = 26	X	X	**AY496727** (re-edited)	**AY496727** (re-edited)	**HM210178**
A. damon	MAT-99-Q841	[h]n = 45	X	X	**AY496732** (new part seq)	AY496732	**HM210179**
A. dantchenkoi	MW99274	[i]n = 42		X	AY557072		AY556678
A. dantchenkoi	MW99276	[e]n = *ca*40-43		X	AY557073		AY556679
A. dantchenkoi	MW99319	[i]n = 42		X	AY557081		AY556685
A. dantchenkoi	MW99320	[e]n = *ca*40-41		X	AY557082		
A. dantchenkoi	VL-01-L342	[i]n = 42	X	X	**AY496737** (re-edited)	**AY496737** (re-edited)	
A. dolus virgilia	RE-07-G106	[k]n = 122	X	X	**HM210162**	**HM210162**	**HM210180**
A. dolus vittatus	MAT-99-Q923	[k]n = 124-125	X	X	**AY496740** (new part seq)	**AY496740** (re-edited)	**HM210181**
A. eriwanensis	AD-00-P303	[j]n = 32	X	X	**AY496742** (re-edited)	**AY496742** (re-edited)	
A. erschoffii	AD-02-L274	[b]n = 13	X	X	**AY496743** (new part seq)	AY496743	**HM210182**
A. exuberans	RE-07-G229	[d]2n = *ca*180	X	X	**HM210172**	**HM210172**	**HM210183**
A. fabressei fabressei	**JM**00001	[a]n = 90		X	AY556869		AY556734
A. fabressei fabressei	MAT-99-Q972	[a]n = 90	X	X	**HM210165**	**HM210165**	**HM210184**
A. fabressei fabressei	MAT-99-Q984	[a]n = 90	X	X	**AY496744** (new part seq)	**AY496744** (re-edited)	**HM210185**
A. fabressei fabressei	MW01039	[a]n = 90		X	AY556952		AY556608
A. fabressei fabressei	RV-03-H596	[a]n = 90	X	X	**EF104605** (re-edited)	**EF104605** (re-edited)	**HM210186**
A. fabressei subbaeticus	RV-03-H554	[d]n = 90					
A. fabressei subbaeticus	RV-03-H555	[d]n = 90	X	X	**HM210166**	**HM210166**	**HM210187**
A. fabressei subbaeticus	RV-03-H556	[d]n = 90					
A. fabressei subbaeticus	RV-03-H557	[d]n = 90					
A. fabressei subbaeticus	RV-03-H558	[d]n = 90	X	X	**EF104604** (re-edited)	**EF104604** (re-edited)	**HM210188**
A. fabressei subbaeticus	RV-03-H560	[d]n = 90					
A. fulgens	MAT-99-Q910	[k]n = 109	X	X	**AY496746** (new part seq)	**AY496746** (re-edited)	**HM210189**
A. fulgens	MW01107	[k]n = 109		X	AY556963		AY556615
A. galloi	RE-07-G436	[d]n = 90	X	X	**HM210167**	**HM210167**	**HM210190**
A. galloi	RE-07-G437	[d]n = 90	X	X	**HM210168**	**HM210168**	**HM210191**
A. galloi	RE-07-G441	[d]n = 90					
A. galloi	RE-07-G445	[d]n = 90					
A. galloi	RE-07-G447	[d]n = 90					
A. humedasae	MW99591			X	AY557127		AY556710 .

Table 2. *Continued*

Taxon (Traditionally) accepted name and combination)	Sample code	Karyotype analysis	Molecular 45-taxa dataset	Molecular 80-taxa dataset	COI genbank code	COII genbank code	ITS2 genebank code
A. humedasae	MW99605			X	AY557128		AY556711
A. humedasae	RE-07-G191	[d]n = 39	X	X	**HM210169**	**HM210169**	**HM210192**
A. humedasae	RE-07-G192	[d]n = 39					
A. humedasae	RE-07-G193	[d]n = 39					
A. humedasae	RE-07-G194	[d]n = 39					
A. humedasae	RE-07-G203		X	X	**HM210170**	**HM210170**	**HM210193**
A. interjectus	MW99164	[e]n = 31		X	AY557059		AY556671
A. khorasanensis	VL-03-F526	[b]n = 84	X	X	AY954013	AY954013	
A. khorasanensis	WE02431			X	AY557138		AY556737
A. menalcas	MW98020			X	AY556982		
A. menalcas	MW98172			X	AY557001		AY556635
A. menalcas	MW99494			X	AY557111		
A. menalcas	VL-01-L122	[b]n = 85	X	X	AY496763	AY496763	**HM210194**
A. ripartii	AD-00-P033		X	X	**AY496787** (re-edited)	**AY496787** (re-edited)	
A. ripartii	**J**C00043			X	AY556858		AY556727
A. ripartii budashkini	NK-00-P859	[l]n = 90	X	X	**AY496779** (re-edited)	**AY496779** (re-edited)	**HM210195**
A. ripartii colemani	NK-00-P822	[m]n = 90	X	X	**AY496781** (re-edited)	**AY496781** (re-edited)	
A. ripartii paralcestis	MW99068	[e]n = *ca*90		X	AY557042		
A. ripartii paralcestis	MW99196			X	AY557064		AY556673
A. ripartii paralcestis	MW99263			X	AY557070		
A. ripartii paralcestis	MW99264			X	AY557071		
A. ripartii paralcestis	AD-00-P337		X	X	**AY496782** (re-edited)	**AY496782** (re-edited)	
A. ripartii paralcestis	VL-01-L103	[b]n = *ca*90	X	X	AY496783	AY496783	
A. ripartii paralcestis	VL-01-L166	[c]n = 90	X	X	AY496784	AY496784	
A. ripartii ripartii	MW01014	[e]n = *ca*90		X	AY556944		AY556603
A. ripartii ripartii	MW01072			X	AY556957		
A. ripartii rippertii	RE-07-G266	[d]n = 90	X	X	**HM210171**	**HM210171**	**HM210196**
A. ripartii rippertii	RE-07-G273	[d]n = 90					
A. ripartii sarkani	NK-00-P829	[m]n = 90	X	X	AY496785	AY496785	
A. ripartii sarkani	NK-00-P848	[m]n = 90	X	X	AY496786	AY496786	
A. ripartii susae	RE-07-G254		X	X	**HM210163**	**HM210163**	**HM210197**
A. ripartii susae	RE-07-G255		X	X	**HM210164**	**HM210164**	**HM210198**
A. rjabovi	VL-02-X474	[b]n = 43	X	X	AY954006	AY954006	
A. rjabovi	VL-03-F816	[b]n = 49	X	X	AY954019	AY954019	
A. surakovi	AD-00-P006	[j]n = 50	X	X	**AY496792** (re-edited)	**AY496792** (re-edited)	**HM210199**
A. urmiaensis	VL-04-E365	[c]n = 19	x	x	**EF104631** (re-edited)	EF104631	
A. valiabadi	MW00064			x	AY556882		AY556557
A. valiabadi	MW00498	[e]n = 23		x	AY556934		AY556594
A. violetae	FGT-05-J629		x	x	**HM210173**	**HM210173**	**HM210200**
A. violetae	FGT-05-J630	[d]n = *ca*90	x	x	**HM210174**	**HM210174**	**HM210201**
A. violetae	RVcoll.08.H299		x	x	**HM210175**	**HM210175**	**HM210202**

[a]The karyotype information for the population studied (but not for this individual) was taken from de Lesse (1960a).
[b]The karyotype of this sample was studied in Lukhtanov *et al.* (2005).
[c]The karyotype of this sample was studied by Lukhtanov (unpublished).
[d]The karyotype of this sample was studied in the present work.
[e]The karyotype of this sample was studied in Wiemers (2003).
[f]The karyotype information for the population studied (but not for the same individual) was taken from Coutsis *et al.* (1999).
[g]The karyotype information for the population studied (but not for the same individual) was taken from Kandul and Lukhtanov (1997).
[h]The karyotype information for the population studied (but not for the same individual) was taken from de Lesse (1960b).
[i]The karyotype of this sample was studied in Lukhtanov *et al.* (2003).
[j]The karyotype information for the population studied (but not for the same individual) was taken from Lukhtanov and Dantchenko (2002b).
[k]The karyotype information for the population studied (but not for the same individual) was taken from Lukhtanov *et al.* (2006).
[l]The karyotype information for the population studied (but not for the same individual) was taken from Kandul *et al.* (2004).
[m]The karyotype of this sample was studied in Lukhtanov and Dantchenko (2002a).

few changes to these were introduced. In two cases, an additional terminal fragment was sequenced using the same specimen. Revised sequences have been updated in GenBank. The final dataset includes 80 specimens representing 37 taxa, including four outgroups. We also analyzed a subset of these taxa: the 45-specimen dataset includes only those samples with little or no missing data.

Sequences were unambiguously aligned using SEQUENCHER, version 3.1 (Genecodes Corporation). For each dataset and gene, regions where more than 50% of the sequences contained missing data were removed using the software GBLOCKS, version 0.91 (Castresana, 2000). The incongruence length difference (ILD) test (Farris *et al.*, 1994) was performed to study the homogeneity between our mitochondrial and nuclear datasets. The test was performed with PAUP* using heuristic searches with tree bisection–reconnection (TBR) branch swapping and 100 random taxon addition replicates, saving no more than ten equally parsimonious trees per replicate. Only parsimony informative sites were included. No significant conflict ($P = 0.98$) was detected by the ILD test between the mitochondrial (COI + tRNALeu + COII) and nuclear (ITS2) data. Thus, we combined mitochondrial and nuclear sequences to improve phylogenetic signal. This resulted in concatenated alignments with a total of 2812 bp for the 45-specimen dataset (mean = 2452 bp, SD = 430.7), and 2691 bp for the 80-specimen dataset (mean = 1843 bp, SD = 788.2).

Phylogenetic relationships were inferred using maximum likelihood (ML), Bayesian Inference (BI) and maximum parsimony (MP). MODELTEST, version 3.6 (Posada & Crandall, 1998) was used to determine substitution models for model-based phylogenetic inferences according to hierarchical likelihood ratio tests (Huelsenbeck & Crandall, 1997).

Maximum likelihood

For ML trees, we used PHYML, version 2.4.4 (Guindon & Gascuel, 2003) with the nucleotide substitution model HKY (Hasegawa, Kishino & Yano, 1985). This software also estimated the Gamma distribution parameter, proportion of invariable sites and nucleotide frequencies. Branch support was assessed using 100 bootstrap replicates.

Bayesian inference

Bayesian analyses were conducted using MRBAYES, version 3.1.2 (Huelsenbeck & Ronquist, 2001). Datasets were partitioned by gene, and by codon position for COI and COII. Substitution models used for each partition were chosen according to MODELTEST (F81 for the second position of COI, GTR for the third position of COI, and HKY for the rest of partitions). Two runs of 1 000 000 generations with four chains (one cold and three heated) were performed. Chains were sampled every 100 generations, and burn-in was determined based on inspection of log likelihood over time plots using TRACER, version 1.4 (available from http://beast.bio.ed.ac.uk/Tracer).

Maximum parsimony

MP analyses were conducted using PAUP, version 4.0b10 (Swofford, 2000). Heuristic searches were performed with TBR branch swapping and 10 000 random taxon addition replicates, saving no more than ten equally parsimonious trees per replicate. To estimate branch support on the recovered topology, nonparametric bootstrap values (Felsenstein, 1985) were assessed with PAUP, version 4.0b10. One hundred bootstrap pseudoreplicates were obtained under a heuristic search with TBR branch swapping with 1000 random taxon addition replicates for the 45 taxon set, saving no more than ten equally parsimonious trees per replicate. Given the long computational time required for the 80-specimen set, 100 random taxon addition replicates were used in this case.

Dating phylogenetic events

BEAST, version 1.4.8 (Drummond & Rambaut, 2007) was used to estimate node ages. The analysis was carried out using the 45-taxa COI and COII dataset, with the same conditions described above for Bayesian phylogeny reconstruction. Monophyly constriction was enforced for several nodes according to the topology in Figure 1. Because no external calibration points, either in the form of a fossil or biogeographic event, are available for *Agrodiaetus*, we used a similar approach to that of Kandul *et al.* (2004). We selected two strongly supported nodes: one within the *dolus* species group and one within the *admetus* species group. Both are of an age close to 0.5 Myr, which we consider adequate to minimize the effects of saturation. Mean uncorrected pairwise distances within the two clades were calculated using MEGA4 (Tamura *et al.*, 2007). Dates for the two calibration points were the arithmetic means of the ages obtained applying a molecular clock with two published substitution rates: 1.5% uncorrected pairwise distance per million years estimated using a variety of invertebrates (Quek *et al.*, 2004) for COI, and a faster rate of 2.3% uncorrected pairwise distance per million years for the entire mitochondrial genome of various arthropod taxa (Brower, 1994). A normal prior distribution was used and the standard deviation was tuned so that the 95% central posterior density included the ages obtained with both rates. The dataset was analyzed under the HKY model applying a strict molecular clock along the branches. Base frequencies were estimated and the site heterogeneity

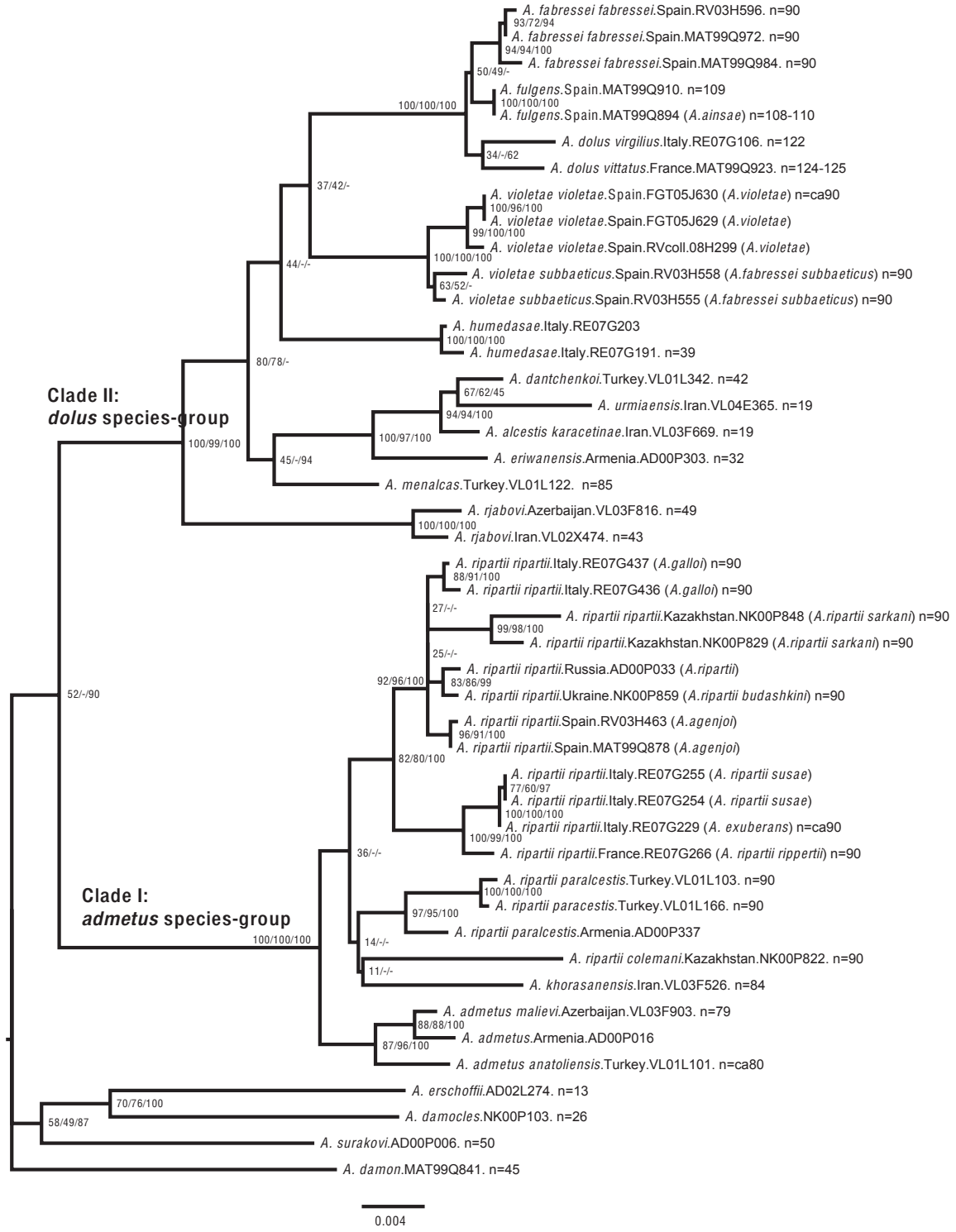


Figure 1. Maximum likelihood tree of *Agrodiaetus* based on the combined analysis of the mitochondrial cytochrome oxidase subunit *I* (COI), leucine transfer RNA (leu-tRNA), cytochrome oxidase subunit *II* (COII) and nuclear internal transcribed spacer 2 (ITS2) (2812 bp) from 45 samples of *Agrodiaetus* according to the Hasegawa, Kishino & Yano model (log likelihood score = –8727.72). Traditional names are indicated in parentheses when new names or combinations are proposed. Haploid chromosome numbers (*n*) are indicated after specimen codes. Numbers at nodes indicate maximum likelihood bootstrap/maximum parsimony bootstrap/Bayesian posterior probability, with nonmatching clades using different analyses indicated by '–'. The scale bar represents 0.004 substitutions/position.

model gamma with four categories was used. Parameters were estimated using two independent runs of 10 million generations each (with a pre-run burn-in of 100 000 generations) to ensure convergence, and checked with the software TRACER, version 1.4. Summary trees were generated using TREEANNOTATOR, version 1.4.8 (available from http://beast.bio.ed.ac.uk).

RESULTS

KARYOTYPES

Karyotype of A. violetae

The taxon *A. violetae* is extremely rare. We were able to obtain a limited number of individuals, of which only one sample had metaphase plates suitable for determination of karyotype characteristics. In this preparation, the chromosome number was determined to be $n = ca90$ (Table 3). Two chromosomes were especially large (Fig. 2A) in the second metaphase of meiosis (MII) complement, and one chromosome was medium-sized. The two largest chromosomes were nearly of equal size, and the medium-sized chromosome was 1.8–2.0 times smaller than these.

Karyotype of A. fabressei subbaeticus

The haploid chromosome number of *A. fabressei subbaeticus* was found to be $n = 90$ (Fig. 2B, C, Table 3), thus confirming our previous results (Lukhtanov *et al.*, 2006). Three bivalents were especially large (Fig. 2B) in the first metaphase of meiosis (MI) complement. Bivalent 1 was only slightly larger than bivalent 2, and the latter was 1.4–1.8 times larger than bivalent 3. In the MII complement, the two largest chromosomes were nearly of equal size, and chromosome 3 was 1.8–2.0 times smaller than the two biggest chromosomes (Fig. 2C).

Karyotype of A. humedasae

The haploid chromosome number was determined to be $n = 39$ (Table 3). Bivalents in MI and chromosomes in MII were fairly differentiated with respect to their size; however, it is difficult to divide them objectively into size groups because the sizes of the 39 bivalents decrease more or less linearly (Fig. 2D, E, F).

KARYOTYPES OF *A. AGENJOI*, *A. RIPARTII* RIPPERTII, *A. GALLOI*, AND *A. EXUBERANS*

The haploid chromosome number was determined to be $n = 90$ in *agenjoi*, *rippertii*, and *galloi*. In MI, two bivalents were especially large and were situated in the centre of the metaphase plates. Bivalent 1 was 1.4–1.6 times larger than bivalent 2. The sizes of the remaining 88 bivalents decreased more or less linearly (Fig. 2G, H, I, J, K, L). Few meiotic metaphase

Table 3. Number of bivalents and mitotic chromosomes observed in the taxa and specimens studied

Taxon	Specimen code number	Country	Haploid (n) or diploid ($2n$) chromosome number	Number of cells with accurately determined bivalent/ chromosome number	Number of large (L) and medium (M) bivalents/chromosomes in haploid complement
violetae	FGT-05-J630	Spain	$n = ca90$	–	2L + 1M
subbaeticus	RV-03-H554	Spain	$n = ca90$	–	2L + 1M
subbaeticus	RV-03-H555	Spain	$n = 90$	5MI	2L + 1M
subbaeticus	RV-03-H556	Spain	$n = ca90$	–	2L + 1M
subbaeticus	RV-03-H557	Spain	$n = 90$	2MII	2L + 1M
subbaeticus	RV-03-H558	Spain	$n = 90$	4MI	2L + 1M
subbaeticus	RV-03-H560	Spain	$n = 90$	2MI, 2MII	2L + 1M
humedasae	RE-7-G191	Italy	$n = 39$	12MI	–
humedasae	RE-7-G192	Italy	$n = 39$	8MI	–
humedasae	RE-7-G193	Italy	$n = 39$	4MII	–
humedasae	RE-7-G194	Italy	$n = 39$	7MI	–
agenjoi	RV-07-F038	Spain	$n = 90$	5MI, 3MII	1L + 1M
rippertii	RE-7-G266	France	$n = 90$	2MI, 2MII	1L + 1M
rippertii	RE-7-G273	France	$n = 90$	3MI	1L + 1M
exuberans	RE-7-G229	Italy	$2n = ca180$	–	1L + 1M
galloi	RE-7-G436	Italy	$n = 90$	7MI, 3MII	1L + 1M
galloi	RE-7-G437	Italy	$n = 90$	6MI, 3MII	1L + 1M
galloi	RE-7-G441	Italy	$n = 90$	4MI	1L + 1M
galloi	RE-7-G445	Italy	$n = 90$	4MI	1L + 1M
galloi	RE-7-G447	Italy	$n = ca90$	–	1L + 1M

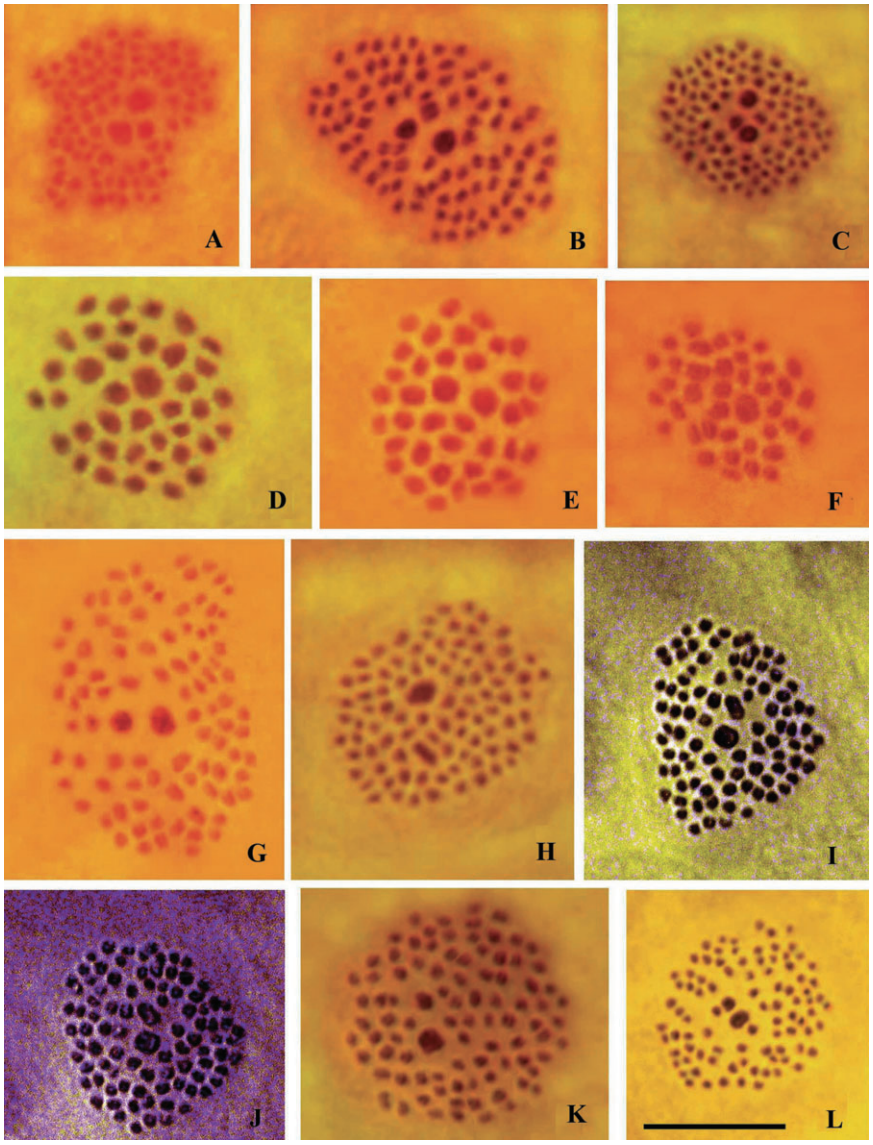


Figure 2. *Agrodiaetus* karyotypes. Scale bar corresponds to 10 mm in all figures. A, *Agrodiaetus violetae violetae* (sample FGT-05-J630). Pole view of a second metaphase of meiosis (MII) plate ($n = ca90$). Two large and one medium-sized chromosome in the centre of the plate can be seen. B, squash preparation of *Agrodiaetus violetae subbaeticus* **comb. nov.** (sample RV-03-H555). First metaphase of meiosis (MI) plate ($n = 90$). Three bivalents are larger than the rest (two large and one medium) in the centre of the metaphase plate. C, *Agrodiaetus violetae subbaeticus* **comb. nov.** (sample RV-03-H560). Pole view of an intact (unsquashed) MII plate ($n = 90$). All the chromosomes are situated in a plane with the largest elements in the centre of the circular metaphase plate clearly separated from each other by gaps. Three chromosomes are larger than the rest (two large + one medium). D, E, F, *Agrodiaetus humedasae*. Pole view of intact (unsquashed) MI plates ($n = 39$). Bivalents are fairly differentiated with respect to their size; however, it is difficult to divide them objectively into size groups because the sizes of the 39 bivalents decrease more or less linearly. D, sample RE-07-G191; E, sample RE-07-G192; F, sample RE-07-G194. G, squash preparation of *Agrodiaetus ripartii agenjoi* (sample RVcoll.07-F038). MI plate ($n = 90$). Two bigger bivalents (one large and one medium) are in the centre of the metaphase plate. H, *Agrodiaetus ripartii rippertii* (sample RE-07-G273). MI plate ($n = 90$). Pole view of a slightly squashed MI plate. Two larger bivalents (one large and one medium) are on the metaphase plate. The original position of the bivalents was altered during preparation, and the medium bivalent is no longer situated in the centre, as it was initially. I, J, K, L, *Agrodiaetus ripartii galloi*. MI plates ($n = 90$). Two bivalents are bigger than the rest (one large and one medium) in the centre of the metaphase plates. I, J, slightly squashed plates of sample RE-07-G436; K, a squashed plate of sample RE-07-G437. L, squash preparation of *Agrodiaetus ripartii galloi* (sample RE-07-G436). MII plate ($n = 90$). Two chromosomes are bigger than the rest (one large and one medium) in the centre of the metaphase plate.

plates were found in *exuberans*, and they were not acceptable for chromosome counts. However, they each displayed one large and one medium bivalent in MI, exactly as it was found in *A. ripartii*. The diploid chromosome number of *exuberans*, however, could be established to be $n = ca180$ (with two larger and two medium-sized chromosomes), which would correspond to a haploid number of $n = ca90$ with one larger and one medium-sized bivalent (Table 3).

PHYLOGENY

Analyses for both the 45-specimen dataset and the 80-specimen dataset recover the *admetus* (clade I) and the *dolus* (clade II) species groups as strongly supported (Figs 1, 3). This concords with results of other studies (Kandul *et al.*, 2002, 2004, 2007; Wiemers, 2003). Within each of these two main groups, many clades are well supported, whereas some of the relationships are not fully resolved. If we compare analyses from the 45-specimen dataset and the 80-specimen dataset, we find that the addition of short COI sequences and ITS2 from Wiemers (2003) adds information by expanding the sampling, but generally produces a lowering of node support. This may be explained by the low overlap of these short COI sequences with many of the longer ones, as well as the low variability of the ITS2 marker between closely-related taxa. Indeed, a tree generated exclusively from ITS2 data (not shown), recovers only the deepest nodes defining the *dolus* and the *admetus* species groups, except for *Agrodiaetus valiabadi*, whose placement is unresolved. Within the *dolus* group, ITS2 supports the *dolus–fulgens–fabressei* clade, the close relationship between the taxa *violetae* and *subbaeticus*, as well as the sister relationship between *A. humedasae* and *Agrodiaetus aroanensis*. Thus, the utility of ITS2 is limited, although, because it is a nuclear marker, it independently confirms the main groups obtained using the mitochondrial data.

Dating analysis (Fig. 4) estimated an age of 3.21 Myr (2.25–4.29; error interval covering 95% highest posterior density) for the genus *Agrodiaetus*, similar to the dates obtained in previous studies (Mensi *et al.*, 1994; Kandul *et al.*, 2004). The estimated age for the split between the sister *dolus* and *ripartii* lineages is 2.73 Myr (range 1.89–3.58 Myr). Finer relationships recovered within each species group and their ages are described in detail in the Discussion, together with their taxonomical implications.

DISCUSSION

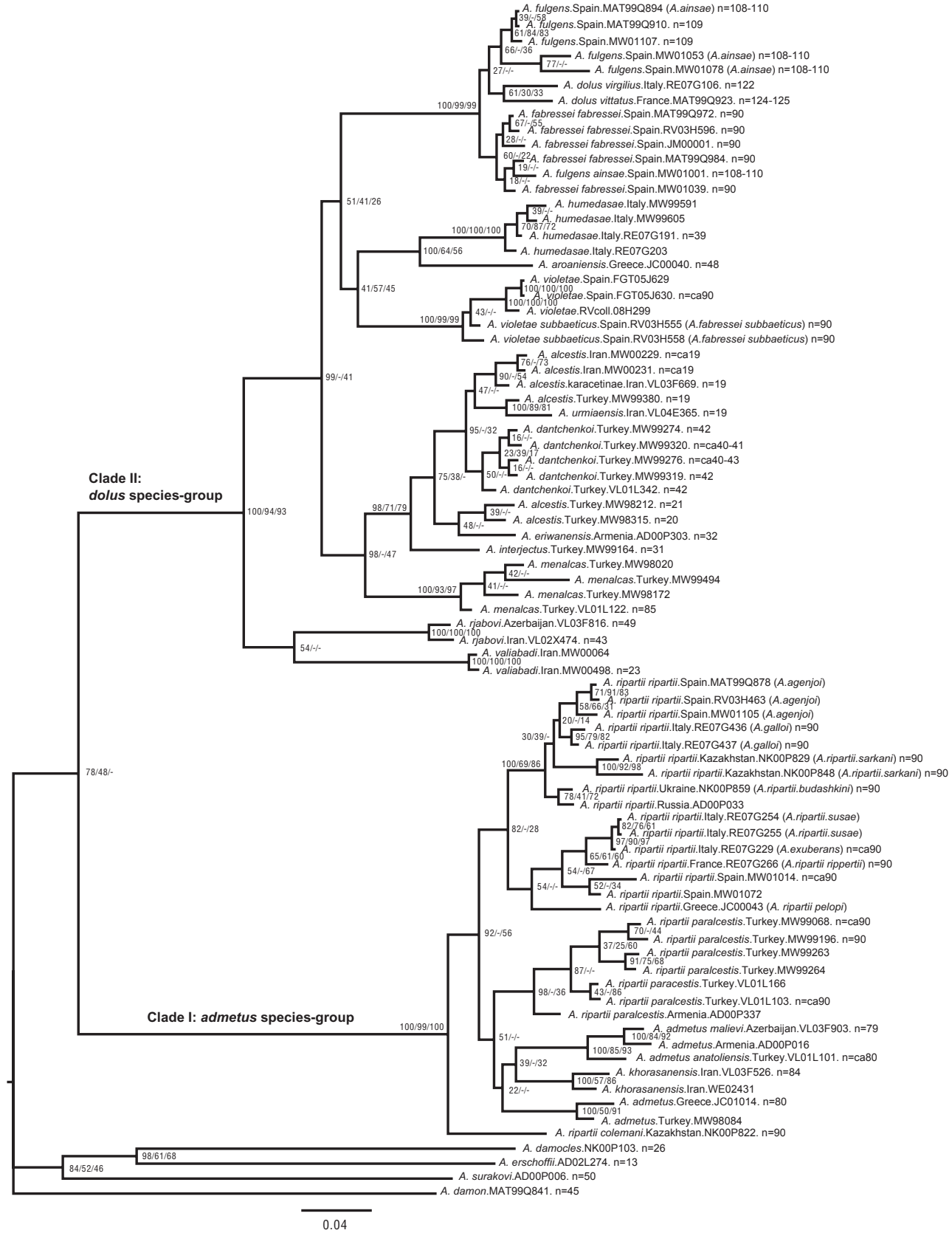
TAXONOMICAL OVERSPLITTING IN WESTERN EUROPEAN AGRODIAETUS

The European *Agrodiaetus* taxa distributed west of the 17th meridian belong to three different phylogenetic lineages (Kandul *et al.*, 2002, 2004, 2007; Wiemers, 2003; our data). One highly differentiated lineage is sister to all other *Agrodiaetus* and consists of a single species, *A. damon*, which has a broad distribution range from Spain to Mongolia (Fig. 5A). This species has no close relatives, and its standing as a good species has never been disputed. All other western European taxa constitute two lineages: the *A. ripartii* lineage, which is part of clade I, and the *A. dolus* lineage, which is part of clade II (Figs 1, 3). The *A. ripartii* lineage includes the taxa *agenjoi*, *exuberans*, *galloi*, *pelopi*, *ripartii*, *rippertii*, and *susae*. The *A. dolus* lineage includes the taxa *ainsae*, *aroaniensis*, *dolus*, *fabressei*, *fulgens*, *humedasae*, *subbaeticus*, *violetae*, *virgilia*, and *vittatus*. The present study supports all previous conclusions about the general taxonomic structure of the *A. admetus* (clade I) and the *A. dolus* (clade II) species groups. At the same time, it sheds light on the taxonomic status and phylogenetic relationships of several western European species whose positions were under debate.

Agrodiaetus ripartii lineage (Fig. 5B)

Agrodiaetus agenjoi: This taxon was described by Forster (1965) from Barcelona (Catalonia, Spain) as a subspecies of the Balkanian–Anatolian species *A. admetus*. Subsequently, de Lesse (1968) and Munguira, Martín & Pérez-Valiente (1995) demonstrated the karyotype similarity of the taxon *agenjoi* and *A. ripartii* (both taxa have $n = 90$, including one large and one medium-sized chromosome pair) and suggested that *A. agenjoi* should be considered a subspecies of *A. ripartii*. Despite these chromosomal studies, and without any explicit justification, *agenjoi* is often treated in the literature as a distinct species with a

Figure 3. Bayesian tree based on the combined analysis of data from mitochondrial cytochrome oxidase subunit *I* (COI), leucine transfer RNA (leu-tRNA), cytochrome oxidase subunit *II* (COII) and nuclear internal transcribed spacer 2 (ITS2) (2691 bp), partitioned by marker and gene codon position, from 80 samples of *Agrodiaetus* (log likelihood score = –7942.31). Traditional names are indicated in parentheses when new names or combinations are proposed. Haploid chromosome numbers (*n*) are indicated after the specimen code numbers. Numbers at nodes indicate Bayesian posterior probability/maximum likelihood bootstrap/maximum parsimony bootstrap, with nonmatching clades among different analysis indicated by '–'. The scale bar represents 0.04 substitutions/position.


A. fulgens.Spain.MAT99Q894 (A.ainsae) n=108-110
A. fulgens.Spain.MAT99Q910. n=109
A. fulgens.Spain.MW01107. n=109
A. fulgens.Spain.MW01053 (A.ainsae) n=108-110
A. fulgens.Spain.MW01078 (A.ainsae) n=108-110
A. dolus virgilius.Italy.RE07G106. n=122
A. dolus vittatus.France.MAT99Q923. n=124-125
A. fabressei fabressei.Spain.MAT99Q972. n=90
A. fabressei fabressei.Spain.RV03H596. n=90
A. fabressei fabressei.Spain.JM00001. n=90
A. fabressei fabressei.Spain.MAT99Q984. n=90
A. fulgens ainsae.Spain.MW01001. n=108-110
A. fabressei fabressei.Spain.MW01039. n=90
A. humedasae.Italy.MW99591
A. humedasae.Italy.MW99605
A. humedasae.Italy.RE07G191. n=39
A. humedasae.Italy.RE07G203
A. aroaniensis.Greece.JC00040. n=48
A. violetae.Spain.FGT05J629
A. violetae.Spain.FGT05J630. n=ca90
A. violetae.RVcoll.08H299
A. violetae subbaeticus.Spain.RV03H555 (A.fabressei subbaeticus) n=90
A. violetae subbaeticus.Spain.RV03H558 (A.fabressei subbaeticus) n=90
A. alcestis.Iran.MW00229. n=ca19
A. alcestis.Iran.MW00231. n=ca19
A. alcestis.karacetinae.Iran.VL03F669. n=19
A. alcestis.Turkey.MW99380. n=19
A. urmiaensis.Iran.VL04E365. n=19
A. dantchenkoi.Turkey.MW99274. n=42
A. dantchenkoi.Turkey.MW99320. n=ca40-41
A. dantchenkoi.Turkey.MW99276. n=ca40-43
A. dantchenkoi.Turkey.MW99319. n=42
A. dantchenkoi.Turkey.VL01L342. n=42
A. alcestis.Turkey.MW98212. n=21
A. alcestis.Turkey.MW98315. n=20
A. eriwanensis.Armenia.AD00P303. n=32
A. interjectus.Turkey.MW99164. n=31
A. menalcas.Turkey.MW98020
A. menalcas.Turkey.MW99494
A. menalcas.Turkey.MW98172
A. menalcas.Turkey.VL01L122. n=85
A. rjabovi.Azerbaijan.VL03F816. n=49
A. rjabovi.Iran.VL02X474. n=43
A. valiabadi.Iran.MW00064
A. valiabadi.Iran.MW00498. n=23
A. ripartii ripartii.Spain.MAT99Q878 (A.agenjoi)
A. ripartii ripartii.Spain.RV03H463 (A.agenjoi)
A. ripartii ripartii.Spain.MW01105 (A.agenjoi)
A. ripartii ripartii.Italy.RE07G436 (A.galloi) n=90
A. ripartii ripartii.Italy.RE07G437 (A.galloi) n=90
A. ripartii ripartii.Kazakhstan.NK00P829 (A.ripartii.sarkani) n=90
A. ripartii ripartii.Kazakhstan.NK00P848 (A.ripartii.sarkani) n=90
A. ripartii ripartii.Ukraine.NK00P859 (A.ripartii.budashkini) n=90
A. ripartii ripartii.Russia.AD00P033
A. ripartii ripartii.Italy.RE07G254 (A.ripartii.susae)
A. ripartii ripartii.Italy.RE07G255 (A.ripartii.susae)
A. ripartii ripartii.Italy.RE07G229 (A.exuberans) n=ca90
A. ripartii ripartii.France.RE07G266 (A.ripartii rippertii) n=90
A. ripartii ripartii.Spain.MW01014. n=ca90
A. ripartii ripartii.Spain.MW01072
A. ripartii ripartii.Greece.JC00043 (A. ripartii pelopi)
A. ripartii paralcestis.Turkey.MW99068. n=ca90
A. ripartii paralcestis.Turkey.MW99196. n=90
A. ripartii paralcestis.Turkey.MW99263
A. ripartii paralcestis.Turkey.MW99264
A. ripartii paracestis.Turkey.VL01L166
A. ripartii paralcestis.Turkey.VL01L103. n=ca90
A. ripartii paralcestis.Armenia.AD00P337
A. admetus malievi.Azerbaijan.VL03F903. n=79
A. admetus.Armenia.AD00P016
A. admetus anatoliensis.Turkey.VL01L101. n=ca80
A. khorasanensis.Iran.VL03F526. n=84
A. khorasanensis.Iran.WE02431
A. admetus.Greece.JC01014. n=80
A. admetus.Turkey.MW98084
A. ripartii colemani.Kazakhstan.NK00P822. n=90
A. damocles.NK00P103. n=26
A. erschoffii.AD02L274. n=13
A. surakovi.AD00P006. n=50
A. damon.MAT99Q841. n=45
Clade II: dolus species-group
Clade I: admetus species-group
0.04

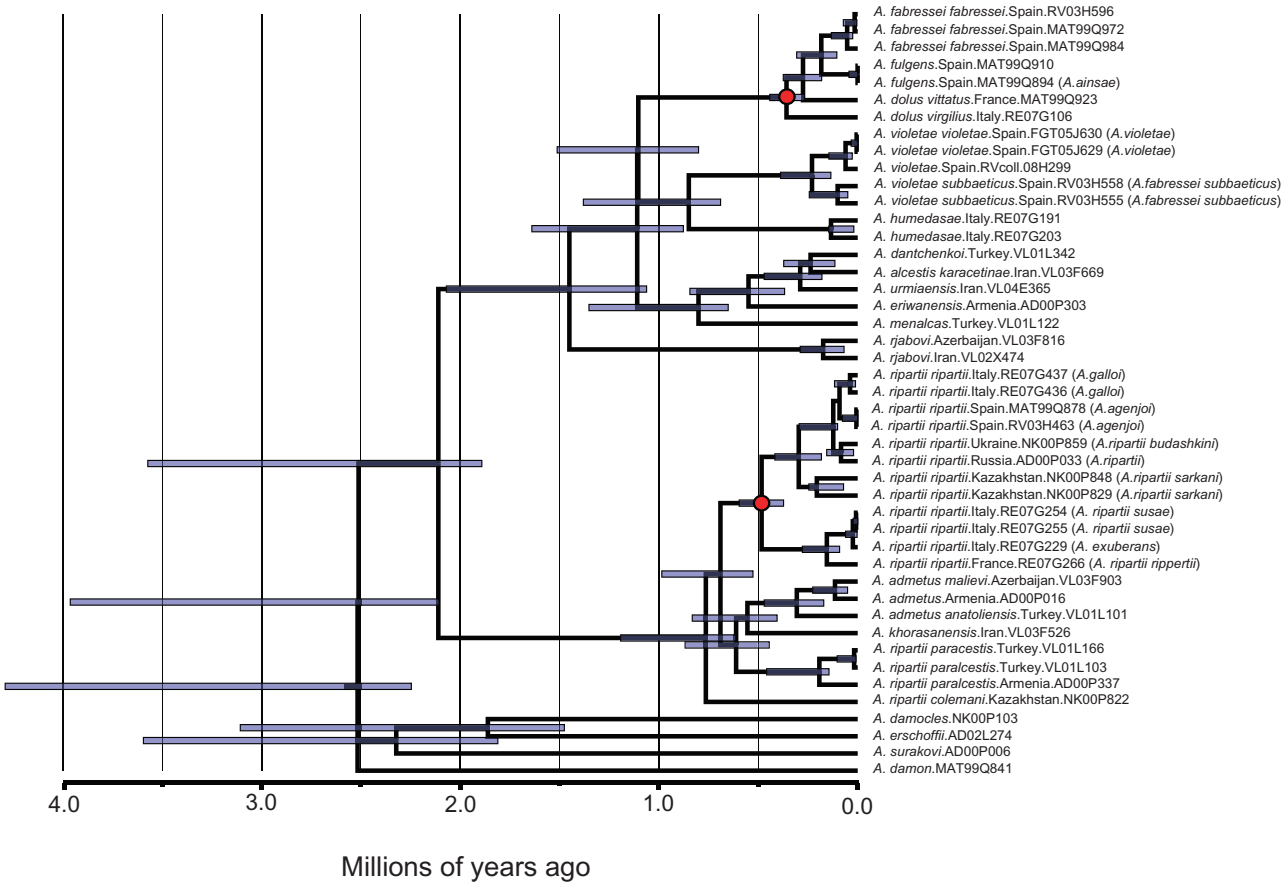


Figure 4. Bayesian ultrametric tree for the 45-taxa dataset obtained with BEAST 1.4.8, based on cytochrome oxidase subunit *I* (COI) and cytochrome oxidase subunit *II* (COII) sequences under the Hasegawa, Kishino & Yano model of DNA substitution. The tree was calibrated at the two nodes indicated (red circles) based on two different published divergence rates for mitochondrial DNA in Arthropoda (1.5% and 2.3% pairwise sequence divergence per million years). For each calibration point, a normal prior distribution was centred on the resulting mean age (and SD) was tuned so that the 95% central posterior density included the ages obtained with both rates. Bars in nodes represent the 95% highest posterior density for age estimations, according to the axis representing time in millions years before present. Traditional names are indicated in parentheses when new names or combinations are proposed.

distributional range restricted to Catalonia in north-east Spain (Kolev & De Prins, 1995; Dennis, 1997; Tolman, 1997; Mazzei *et al.*, 2009) or as a subspecies of *A. fabressei* (Manley & Allcard, 1970) (but see also Munguira *et al.*, (1995) and Eckweiler & Häuser (1997), who considered this taxon a subspecies of *A. ripartii*).

Our molecular phylogeny recovers *A. agenjoi* as an internal clade within one of the *A. ripartii* clades. The monophyly of the *agenjoi* clade has good support in the 45-specimen set, but lower support in the 80-specimen set. Its genetic divergence with respect to *A. ripartii* samples from Russia and Ukraine, as well as with the taxon *A. galloi*, is minimal (0.28–0.56%) and includes only three fixed nucleotide substitutions in 1858 bp of COI-tRNALeu-COII. This difference is extremely small and could even be less when additional individuals and intermediate populations are studied. Our chromosomal data confirm that the karyotype of *A. agenjoi* is indistinguishable from that of *A. ripartii*, and do not support the species status of *A. agenjoi*. Moreover, morphological differences between *A. ripartii* and *A. agenjoi* are subtle and inconstant. The character that is usually used to distinguish between them) the presence of a white stripe on the underside of the hind wing of *A. ripartii*, and its absence in *A. agenjoi*; Tolman, 1997) can be variable in *Agrodiaetus* at the species, population, and individual levels, and its taxonomic significance is also low (Eckweiler & Häuser, 1997; Lukhtanov & Budashkin, 2007). Moreover, although generally absent in *agenjoi*, this streak is present in a low percentage of the Catalonian specimens. Because

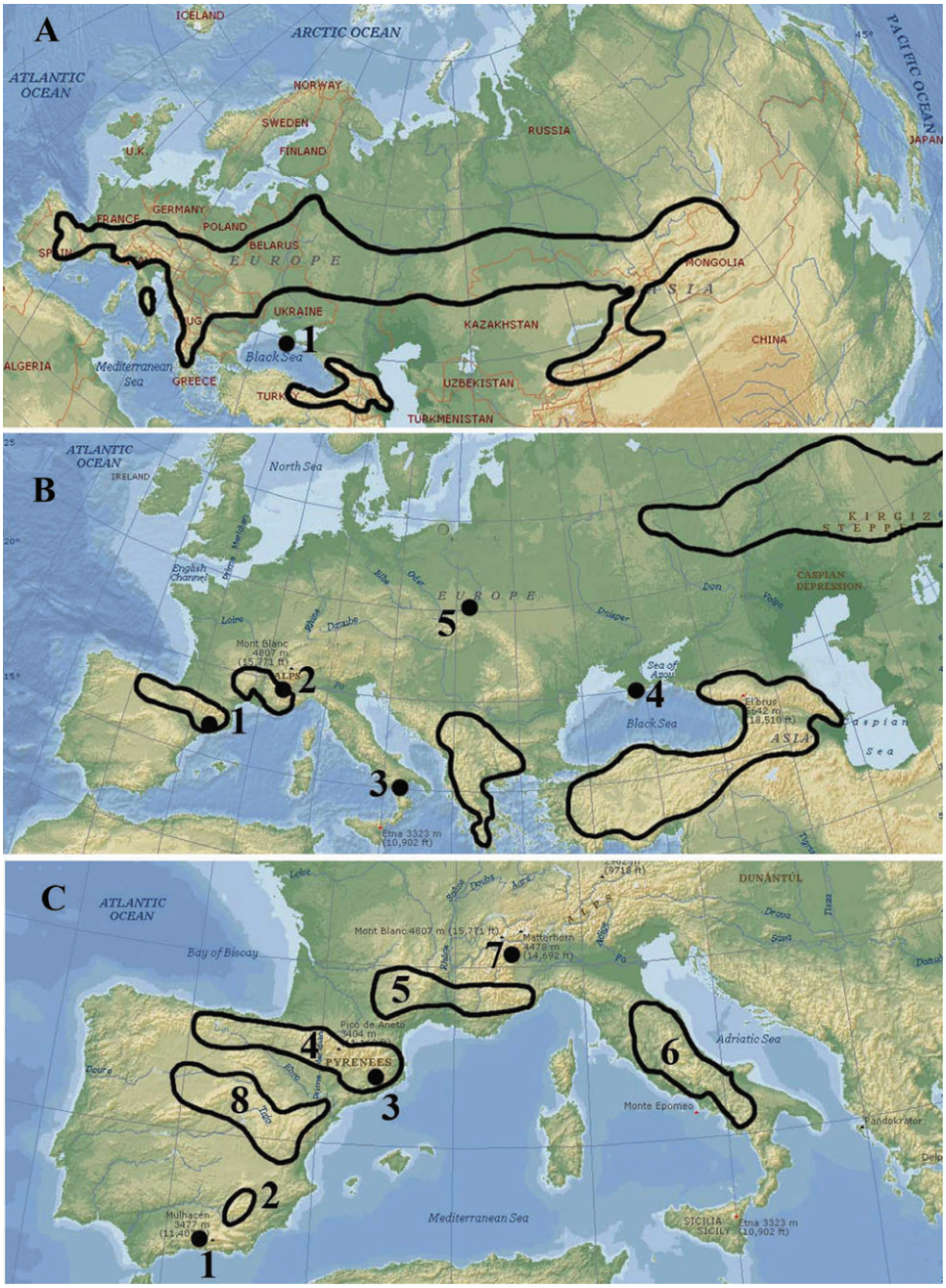


Figure 5. Distribution ranges of western European *Agrodiaetus*, according to data original to the present study, Hesselbarth, Oorchot & Wagener (1995), Kudrna (2002) and García-Barros *et al.* (2004). A, distribution ranges *of Agrodiaetus damon* (closed loops) and *Agrodiaetus pljushtchi* (1). B, distribution ranges of taxa belonging to the *Agrodiaetus ripartii* lineage: 1 – *Agrodiaetus agenjoi* (here assigned to *Agrodiaetus ripartii ripartii*); 2 – *Agrodiaetus exuberans* (here assigned to *Agrodiaetus ripartii ripartii*); 3 – *Agrodiaetus galloi* (here assigned to *A. ripartii ripartii*); 4 – *Agrodiaetus budashkini* (here assigned to *A. ripartii ripartii*); 5 – a geographically isolated population of *A. ripartii* in Poland (Przybylowicz, 2000). Distribution range of the main populations of *A. ripartii* indicated by closed loops. C, distribution ranges of taxa belonging to the *Agrodiaetus dolus* lineage: 1 – *Agrodiaetus violetae violetae*; 2 – *Agrodiaetus violetae subbaeticus* **comb. nov.**; 3 – presumed distribution range of *Agrodiaetus fulgens* before the chromosomal study by Lukhtanov *et al.* (2006); 4 – revised distribution range of *A. fulgens*; 5 – *Agrodiaetus dolus dolus* and *Agrodiaetus dolus vittatus*; 6 – *Agrodiaetus dolus virgilia*; 7 – *Agrodiaetus humedasae*; 8 – *Agrodiaetus fabressei*.

the taxa *ripartii* and *agenjoi* were both described from northern Spain, we consider the name *agenjoi* to be a synonym of *A. ripartii*.

Agrodiaetus galloi: This taxon was described as a distinct species (Baletto & Toso, 1979) from southern Italy on the basis of an extreme difference in karyotype; its chromosome number was established to be $2n = 132$ ($n = 66$), including one pair of large and one pair of medium-sized chromosomes (Troiano & Giribaldi, 1979), whereas *A. ripartii*, geographically and phenotypically the most closely related taxon, has $n = 90$ (de Lesse, 1960b). *Agrodiaetus galloi* has invariably been considered a good species in all studies on European butterflies, with the exception of Eckweiler & Häuser (1997), who questioned the species status of this taxon.

The present study confirms the presence of one large and one medium-sized bivalent in *A. galloi*, but we were unable to confirm the previously reported chromosome number. Without exception, all studied cells and individuals possessed a chromosome number of $n = 90$ (Fig. 2I, J, K, L). We consider that the discrepancy between the earlier chromosome count and ours arises because true MI or MII metaphase cells were not observed in the study by Troiano & Giribaldi (1979). According to their figure 7, which was originally interpreted to be a picture of anaphase I, they in fact observed atypical meiotic divisions. Such atypical divisions occur regularly during male meiosis in all species of Lepidoptera, to the point where during the imaginal stage, they are much more frequent than normal meiotic divisions (Lorkovic, 1990). Generally, atypical divisions display the diploid set; however, the great majority of atypical spermatocytes are not suitable for chromosome counts as a result of multiple nonspecific chromosome conglutinations (Lorkovic, 1990) that can lead to a strong underestimation of the real chromosome number. Thus, we consider that the true number of chromosomes in the samples studied by Troiano & Giribaldi (1979) was also $n = 90$, and that the karyotype of *A. galloi* is in fact indistinguishable from that found in *A. ripartii*.

In our phylogenetic reconstruction, the taxon *galloi* forms a well-supported cluster with *A. ripartii* samples Ukraine, Russia, and Kazakhstan, as well as with the taxon *agenjoi* (Figs 1, 3). Moreover, the genetic divergence of the two studied individuals of *galloi* with respect to the most closely-related *ripartii* and *agenjoi* samples is extremely small and could be even less when additional individuals are studied. The specimens studied have ITS2 sequences identical to those of several *A. ripartii*, excluding the possibility that *galloi* is a diverged taxon that has undergone mitochondrial introgression from *A. ripartii*. Thus, the morphological, chromosomal, and genetic data do not support the treatment of *A. galloi* as a separate species. It should be synonymized with *A. ripartii* or, at most, considered a weakly differentiated local subspecies of *A. ripartii*.

The taxa rippertii, exuberans *and* susae*:* The taxon *rippertii* was described from southern France ('aux environs de Digne') as a separate species by Boisduval (1832). In the original description, however, Boisduval made no reference to Freyer (1830), who established from Spain a morphologically very similar taxon (*ripartii*) 2 years earlier. Therefore, the taxon *rippertii* has been considered a synonym or subspecies of *A. ripartii* in recent literature (Eckweiler & Häuser, 1997).

The taxon *exuberans* was described from 'Oulx' (northern Italy) as a 'race' (i.e. subspecies) of *A. admetus* by Verity (1926). It is similar morphologically to *A. ripartii* and was regarded later as a subspecies or even a synonym of *A. ripartii* (Eckweiler & Häuser, 1997). However, without explicit justification, it has been raised to species rank in most recent studies (Kudrna, 2002; Bertaccini, 2003; Dennis *et al.*, 2008).

The taxon *susae* was described from northern Italy as separate subspecies of *A. ripartii* (Bertaccini, 2003). In accordance with the original description, the taxon *susae* is sympatric with *A. exuberans*, and these two taxa are different in small details of genitalic structure and wing spots. We collected both *exuberans* and *susae* in their exact type locality, and comparison of these individuals showed that the morphological differences between *exuberans* and *susae* are sufficiently subtle so that it is not always possible to distinguish between them in practice (R. Vila & V. A. Lukhtanov, unpubl. observ.). Molecular analysis demonstrated that the taxa *exuberans* and *susae* are almost identical, and genetically similar to *A. ripartii rippertii* from France. These three taxa constitute a well-supported monophyletic clade within the bigger *A. ripartii* clade in the 45-specimen dataset (Fig. 1), although the support of this clade is relatively low in the 80-specimen dataset (Fig. 3). Moreover, nuclear ITS2 sequences of these three taxa are identical, which independently supports the results of the mitochondrial sequences. Chromosomal analysis showed that karyotypes of the taxa *rippertii* and *exuberans* are indistinguishable from those of *A. ripartii* from Europe, Turkey, and Kazakhstan (de Lesse, 1960b; Lukhtanov & Dantchenko, 2002b). We were unable to obtain countable metaphase plates for the taxon *susae* but, taking into account its genetic and morphological similarity to the taxa *rippertii* and *exuberans*, we consider it unlikely to be a separate taxon.

Infraspecific taxonomy of A. ripartii*:* In the molecular phylogeny, *A. ripartii* samples from Asia Minor and Armenia (*Agrodiaetus ripartii paralcestis*) and especially from Central Asia (*Agrodiaetus ripartii colemani*) are genetically distant from European and other Kazakhstani populations (Figs 1, 3). We will not discuss these further here because this is beyond the scope of the present study and our material from these regions is limited. However, all other samples of *A. ripartii* from western Europe, the Balkan Peninsula, European Russia, and the Ukraine (including representatives of the nominal taxa *Agrodiaetus ripartii ripartii*, *A. ripartii rippertii*, *Agrodiaetus ripartii sarkani*, *Agrodiaetus ripartii budashkini*, *A. ripartii susae*, *Agrodiaetus ripartii pelopi*, *A. agenjoi*, *A. exuberans*, and *A. galloi*) form a well-supported clade. The genetically related representatives of this clade display allopatric distributions, are similar in their morphology, and are indistinguishable with respect to karyotype. A more detailed study of *A. ripartii* will be necessary to shed light on relationships between populations and on the total number of subspecies. Given the data available, and until these relationships can be clarified, we provisionally consider all European and North and East Kazakhstani populations to belong to the nominative subspecies *A. ripartii ripartii*. Thus, we recognize three subspecies defined by the main three *A. ripartii* clades: *A. ripartii ripartii*, *A. ripartii paralcestis* and *A. ripartii colemani*.

Agrodiaetus dolus lineage

By contrast to the *A. ripartii* lineage, the *A. dolus* complex is represented in western Europe by a number of distinct taxa that appear to be allopatric in their distribution. All of them are clearly separated from one another by significant chromosomal and/or genetic gaps. Interestingly, two species, *A. dolus and A. fulgens*, are whitish–blue on the upperside of the male wing, and are therefore morphologically different from the rest.

The taxa violetae *and* subbaeticus*:* The taxon *violetae* was described from southern Spain as a new species that is similar to *A. fabresseii*, but differs by the presence of a white stripe on the underside of the hind wing (Gómez-Bustillo, Expósita Hermosa & Martínez Borrego, 1979). The latter character, as already discussed, has low taxonomic significance. The taxon *violetae* is considered in the current literature to be either a valid species (Kudrna, 2002; Gil-T. & Gil-Uceda, 2005; Lafranchis *et al.*, 2007; Gil-T., 2008), a subspecies of *A. fabressei* (Munguira *et al.*, 1995; Eckweiler & Häuser, 1997), a possible subspecies of *A. ripartii* (Tolman, 1997) or a taxon *incertae sedis* (Lukhtanov *et al.*, 2006).

The taxon *subbaeticus* was recently described from southern Spain as a subspecies of *A. fabressei* (Gil-T. & Gil-Uceda, 2005), and its presumed conspecific relationship with *A. fabressei* is supported by chromosomal data (Lukhtanov *et al.*, 2006). In the present study, we analyse for the first time the karyotype of *A. violetae* from the type locality, and show that it is similar to that of the karyotypes of *subbaeticus* and *fabressei*. Thus, from the point of view of karyology, the species status of *A. violetae* is not supported. Our phylogenetic analysis showed that *A. violetae* is unexpectedly quite distant from *Agrodiaetus fabressei fabressei*: these two species are not even sister taxa (Figs 1, 3). On the other hand, the taxa *violetae* and *subbaeticus* form a distinct, highly supported (99–100% bootstrap and BI support) monophyletic clade in all reconstructions. Importantly, the taxa *violetae* and *subbaeticus* have identical ITS2 sequences, and these are quite different from that of *A. fabressei fabressei*. Thus, both nuclear and mitochondrial sequences agree in the close relationship between *violetae* and *subbaeticus*. These results suggest that the taxon *violetae* is a separate species that includes at least two subspecies: *Agrodiaetus violetae violetae* and *Agrodiaetus violetae subbaeticus* **comb. nov.** The subspecific status of *subbaeticus* with respect to *A. violetae* from the type locality is based on morphological differences in the adults (intensity of wing underside spots and female background colour), and in the caterpillars (different colour of the lateral stripes) (Gil-T. & Gil-Uceda, 2005; Gil-T., 2008). These two taxa are allopatric and feed on different subspecies of *Onobrychis argentea* Boiss. (Lafranchis, Gil-T. & Lafranchis, 2007). The present study includes one specimen of a newly discovered, isolated population of *A. violetae*, which is located in a mountain approximately 100 km far from the type locality, and approximately 100 km far from *A. violetae subbaeticus* populations. This population is genetically closer to *A. violetae violetae* and its discovery and status will be described in a future publication (S. Ibáñez & F. Gil-T., unpubl. data). We thus conclude that *A. violetae* is a good local species whose distribution in the south of the Iberian Peninsula is not dot-like, but substantially wider than previously believed.

The taxa dolus *and* virgilia*:* On the basis of karyotype analysis, *A. dolus* consists of two populations with a minor but fixed chromosomal difference between them: the populations from France (*Agrodiaetus dolus dolus* and *Agrodiaetus dolus vittatus*) have $n = 123$–125, with a modal chromosome number of 124, and the populations from central Italy (*Agrodiaetus dolus virgilia*) have $n = 122$ (de Lesse, 1966). Usually, populations are considered to be conspecific. However,

sometimes they are treated as separate species, as in *A. dolus* ($n = 123–125$) and *A. virgilia* ($n = 122$) (de Prins & Iversen, 1996; Dennis, 1997). *A. dolus vittatus* and *A. dolus virgilia* are recovered as sister taxa in our phylogenetic analysis. Although their genetic divergence is intermediate and larger than the *fabressei–fulgens* divergence, the fixed difference in one or two chromosome pairs seems at present insufficient to separate *virgilia* from *dolus* at the species level. Given our current knowledge of reproductive isolation between populations of Lepidoptera with variable karyotypes (Lukhtanov & Dantchenko, 2002b), we consider it more likely that these chromosomal forms are still interfertile.

The taxa ainsae *and* fulgens*:* These two taxa were already shown to be conspecific based on the lack of genetic or karyotypic differences, with similar morphology and ecology (Lukhtanov *et al.*, 2006). The taxon *ainsae* was then considered to be a subspecies of *fulgens* because of small morphological differences, including a higher percentage of specimens with a white band on the underside hindwing and slightly paler male uppersides. However, many new populations between the two type localities have been discovered, and it is difficult to draw a line that defines two subspecies. It appears that a cline exists involving intensity and prevalence of the characters mentioned, and that it probably extends to the west to include the taxa *pseudovirgilius* de Lesse, 1962 and *leonensis* Verhulst, 2004 (not studied here). We thus consider *ainsae* to be a junior subjective synonym of *fulgens*.

Agrodiaetus humedasae*:* This taxon was described from N. Italy (Toso & Balletto, 1976). Its karyotype was found to be $n = 38$ (Troiano, Balletto & Toso, 1979), which is different from that of other representatives of the *A. dolus* and *A. admetus* species groups. Therefore, *A. humedasae* has almost always considered a distinct species. The present study slightly modifies the chromosome number of *A. humedasae* to $n = 39$. In the molecular phylogeny, *A. humedasae* samples form a monophyletic and genetically well-differentiated clade, which is sister to *A. aroaniensis* from Greece (Fig. 3). Interestingly, *A. aroaniensis* also has a relatively low chromosome number ($n = 48$) (Coutsis, Puplesiene & De Prins, 1999). The fact that these two allopatric taxa are chromosomally distinct supports their status as separate species.

PHYLOGEOGRAPHY

A comparison of the distribution ranges of the *A. dolus* and *A. riparii* lineages reveals an interesting pattern (Fig. 5B, C). These two complexes are represented by two groups of geographical isolates with similar population distributions: each lineage has one isolate in the Balkan and Apennine Peninsulas, one isolate in the southern Alps, and from one to four isolates in the Iberian Peninsula. Such a pattern could be considered evidence for similar ecological preferences or parallel histories for these groups. The last assumption may be easily refuted: a comparison of branch lengths on the phylogenetic reconstructions as well as the dating of relevant nodes show that the isolates of these two groups are of different ages and are likely to have originated at different periods of the Pleistocene.

Analysis of distribution and phylogeny in the *A. dolus* lineage shows that the phylogeograpic history of this complex involved a combination of dispersal and vicariance events with a clear general trend of dispersal from the East (Iran), where the group most likely arose, to the West (western Europe) (Fig. 6): The first split, approximately 1.55 Mya (range 1.06–2.07 Mya; error interval covering 95% highest posterior density), was between the Iranian lineage and the rest; the second split, approximately 1.24 Mya (range 0.88–1.64 Mya), was between the Anatolian and European lineages. After this, the European lineage probably spread throughout southern Europe, and approximately 1.15 Mya (range 0.80–1.51 Mya), separated into three clades located in the Balkan Mountains and Alps, southern Spain, and the Iberian–Italian region, respectively. The relatively early separation between the main clades within the *A. dolus* group is in good agreement with their high level of karyotype divergence: the clade had time to develop different chromosome numbers from $n = 39$ in *A. humedasae* to $n = 125$ in *A. dolus*. However, it is interesting to note that the speciation of the taxa *dolus*, *fulgens*, and *fabressei* occurred as recently as 0.36 Mya (range 0.27–0.44 Mya). We specifically discuss the possible origins of these three species below.

Although the *A. ripartii* lineage also has a clear Asian origin, its phylogeographic history seems quite different, especially since it appears to have entered and dispersed in Europe more recently, approximately 0.76 Mya (range 0.53–0.99 Mya). Genetic distance (and correspondingly divergence age) is much lower between *A. ripartii* isolates (Fig. 7). The time of origin of the main *A. ripartii* lineages in Europe and NW Asia can be estimated as approximately 0.48 Mya (range 0.37–0.60 Mya). The alleles of the *COI* gene in the Spanish and Russian- northern Kazakhstani lineages show no lineage sorting, and samples from Spanish populations belong to different haplotype groups (e.g. MW01105 and MW01014; Fig. 3). This absence of lineage sorting can be explained not only by relatively recent origin of lineages, but also by introgression

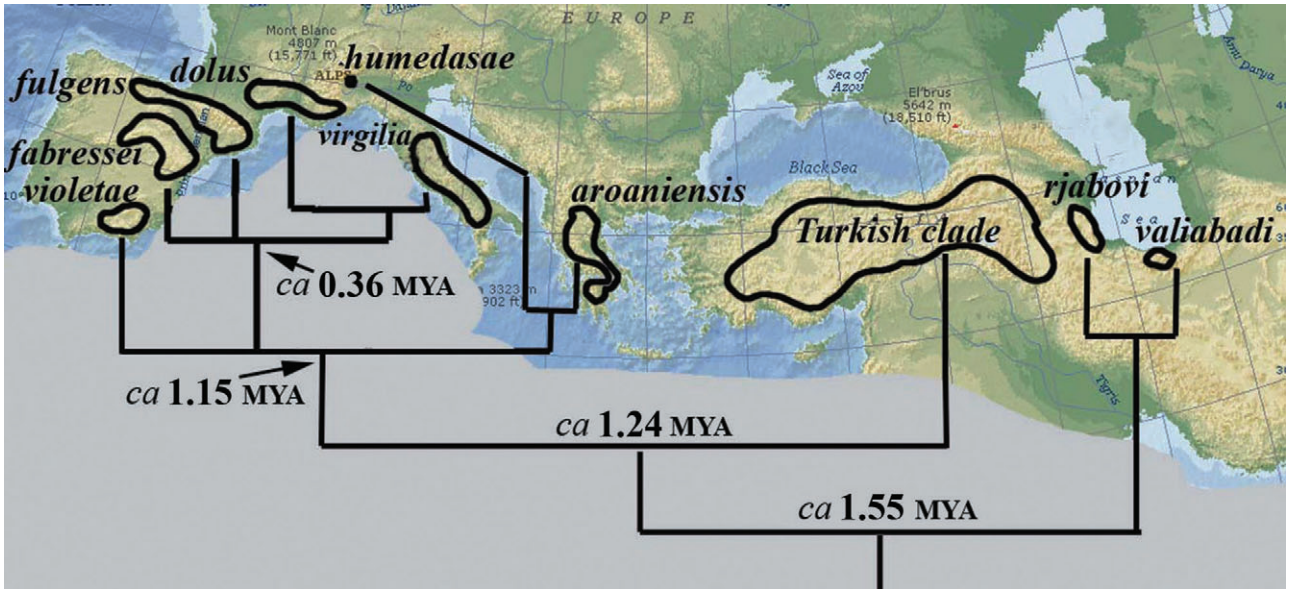


Figure 6. Biogeographical hypothesis describing the first split of the *Agrodiaetus dolus* lineage in the Iranian–Anatolian region, dispersal to Europe and diversification in southern Europe during the Pleistocene.

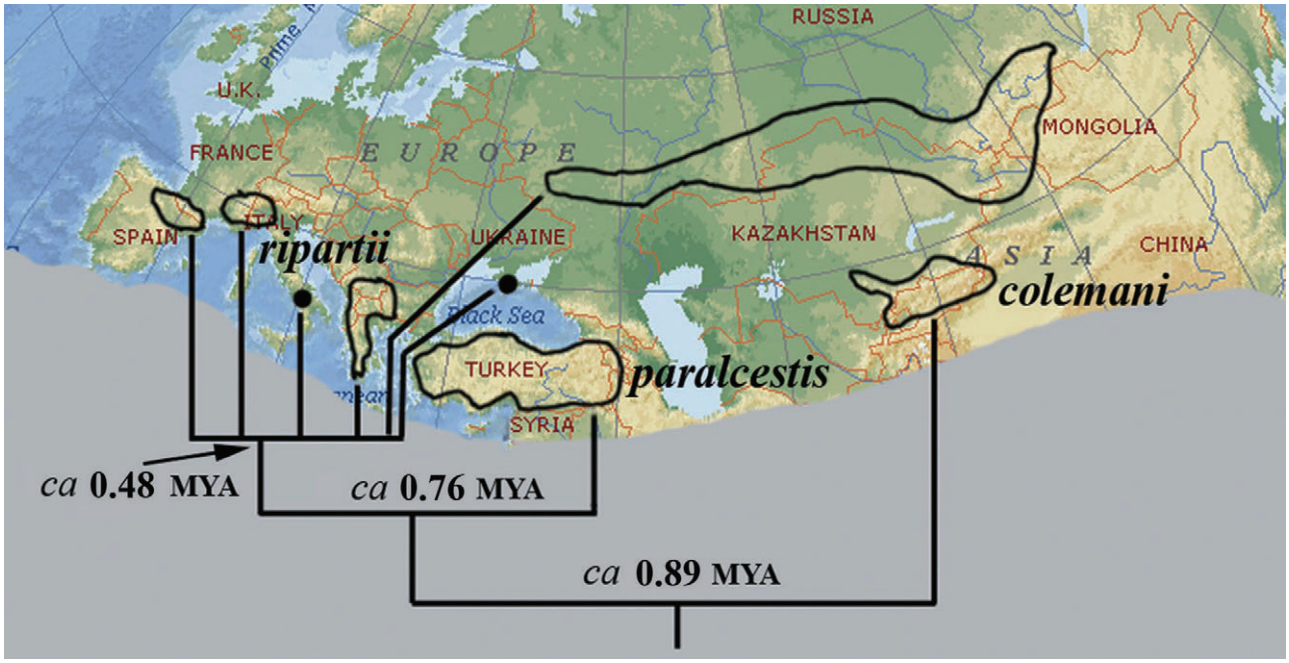


Figure 7. Biogeographical hypothesis describing the first splits of the *Agrodiaetus ripartii* lineage in Asia in the late Pleistocene, and dispersal to Europe and north-west Asia, followed by distribution range fragmentation.

events. Additional indirect evidence supporting the recent divergence hypothesis is the fact that all the clades of the European *A. ripartii* lineage are karyotypically undifferentiated. To conclude, it appears most likely that when *A. ripartii* reached Europe, the Balkan, Apennine and Iberian Peninsulas were already populated by representatives of the *A. dolus* group. Our taxonomic conclusions reflect this difference in biogeographic histories: the older *A. dolus* lineage is represented in western Europe by several species, whereas the younger *A. ripartii* lineage is represented by a group of conspecific populations.

Dot-like distribution ranges and conservation

Our taxonomical revision based on chromosomal and molecular data supports the species status (and consequently the dot-like distribution) of *A. humedasae*. An earlier study likewise supported the dot-like distribution range for *A. pljushtchi* from Crimea (Fig. 5A) (Lukhtanov & Budashkin, 2007). By contrast, we were unable to confirm dot-like distributions for the rest of the studied taxa. The taxa *galloi*, *exuberans*, and *agenjoi* most likely represent local populations of a single species, *A. ripartii*. The same conclusion was earlier obtained (and supported here) for *A. budashkini*, which was described and considered a distinct species from Crimea, but in fact represents an isolated population of *A. ripartii* that is most closely related to the populations in European Russia (Fig. 5B) (Kandul *et al.*, 2004).

Current evidence also supports *A. violetae* as a fairly restricted, good species, but without a dot-like distribution because it consists of at least three groups of populations located in different mountains in the south of the Iberian Peninsula (Fig. 5C). A similar situation was found for *A. fulgens*, which was once considered a species with a very restricted distribution, but later shown to be conspecific with *A. ainsae* (Lukhtanov *et al.*, 2006). Thus, *A. fulgens* must be considered a species with a relatively broad distribution in northern Spain.

In conclusion, of the initial 11 potential cases of dot-like distributed *Agrodiaetus* species in Europe, six are not supported (*A. agenjoi*, *A. budashkini*, *A. exuberans*, *A. fulgens*, *A. galloi*, and *A. violetae*), two are supported (*A. humedasae* and *A. pljushtchi*), and three Balkan taxa remain to be analyzed (*A. nephohiptamenos*, *A. eleniae*, and *A. orphicus*).

Among the studied species, the taxa *A. violetae*, *A. galloi*, and *A. humedasae* are listed as species of conservation concern (Van Swaay *et al.*, 2010) because of their restricted distribution ranges. Two of them (*A. galloi* and *A. humedasae*) are also included in both the IUCN Red List of Threatened Species ((http://www.iucnredlist.org/apps/redlist/details/17939/0; http://www.iucnredlist.org/apps/redlist/details/17941/0) and in the Bern Convention on the Conservation of European Wildlife and Natural Habitats (http://conventions.coe.int/treaty/FR/Treaties/Html/104-2.htm). The results of the present study support the inclusion of *A. humedasae* on these lists. As for *A. galloi*, we show that this taxon is a population of the widely distributed *A. ripartii*, rather than a separate species. This population is geographically strongly isolated and may nevertheless be an important unit for conservation purposes. However, in the light of the data obtained, it is questionable whether it should be prioritized on protection lists above other endangered species.

The classical effect of incorrect taxonomy on conservation efforts is to underestimate the level of biological diversity and, as a consequence, to fail to recognize important conservation units in time (Duagherty *et al.*, 1990; DeSalle & Amato, 2004). By contrast, the present study illustrates a case of overestimation of biological diversity, leading to an inflated number of protected species. This has direct implications for conservation efforts because the protection of invalid species can result in inequitable spending of resources, which are always limited, and divert the attention of biologists and politicians away from species that require more urgent protection.

Species are important practical units in evolution, ecology and conservation, and a complete list of species existing in nature is a fundamental requirement of biodiversity-related studies and their application in all fields of biology. However, every species list contains uncertainties as a result of (1) the evolutionary nature of species, (2) subjectivity in species delimitation, and (3) imperfect taxonomy (Isaac, Mallet & Mace, 2004). The uncertainties of the first type depend on the continuous process of Darwinian evolution giving rise to intermediate forms, or incipient taxa that fail to meet unambiguous criteria for species delimitation (Descimon & Mallet, 2009). The uncertainties of the second type reflect the fact that species have been described and species lists have been created in different taxonomic cultures using different species concepts. These lists are particularly badly affected by extremes of 'splitter' or 'lumper' approaches (Isaac *et al.*, 2004). The first two types of uncertainties are inherent properties of species lists that can probably never be truly eliminated, although species lists can be made more useful if ambiguities are minimized. The third factor, imperfect taxonomy, should in theory be the easiest to uncover, although it frequently results in self-perpetuating error cascades in biological sciences and conservation efforts (Bortolus, 2008). Cases of imperfect taxonomy are unfortunately not rare, even among popular groups such as butterflies, and we advocate that lists of protected butterflies deserve careful revision with the use of modern techniques and consistently applied criteria for species recognition.

Taxonomic conclusion

We propose the following taxonomic arrangement of European representatives (west of the 17th meridian) of the *A. dolus* and *A. ripartii* lineages (chromosome numbers in parentheses when known):

<u>*A. dolus* lineage:</u>
A. dolus (Hübner, [1823])

ssp. *dolus* (Hübner, [1823]) (*n* = 123–125)
ssp. *vittatus* (Oberthür, 1892) (*n* = 124–125)
ssp. *virgilia* (Oberthür, 1910) (*n* = 122)
ssp. *gargano* (Wimmers, 1931) (*n* = 122) (not studied in this paper, probably a synonym of *virgilia*)
ssp. *paravirgilia* Verity, 1943 (*n* unknown) (not studied in this paper, probably a synonym of *virgilia*)
A. fulgens (Sagarra, 1925) (*n* = 108–110) (= *ainsae* Forster, 1961)
taxon *pseudovirgilius* de Lesse, 1962 (*n* = 108) (= *magnabrillata* Gómez-Bustillo, 1971) (not studied in the present study, probably a synonym of *fulgens*)
taxon *leonensis* Verhulst, 2004 (*n* unknown) (not included in the present study, probably a synonym of *fulgens*)
A. fabressei (Oberthür, 1910) (*n* = 90)
A. violetae Gómez-Bustillo *et al.*, 1979
ssp. *violetae* Gómez-Bustillo *et al.*, 1979 (*n* = 90)
ssp. *subbaeticus* Gil-T. & Gil-Uceda, 2005 (*n* = 90)
A. humedasae Toso & Balletto, 1976 (*n* = 39)

A. ripartii lineage:
A. ripartii Freyer, 1830
ssp. *ripartii* Freyer, 1830 (= *agenjoi* Forster, 1965; = *budashkini* Kolev & de Prins, 1995; = *exuberans* Verity, 1926; = *montanesa* Gómez-Bustillo, 1971; = *mozuelica* Agenjo, 1973; = *pelopi* Brown, 1976; = *ramonagenjo* Koçak & Kemal, 2001; = *rippertii* Boisduval, 1832; = *sarkani* Lukhtanov & Dantchenko, 2002; = *susae* Bertaccini, 2003) (*n* = 90)

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