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High molecular and phenotypic diversity in the *Merodon avidus* complex (Diptera, Syrphidae): cryptic speciation in a diverse insect taxon

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This paper examines molecular and phenotypic variability in the widely spread European hoverfly species complex *Merodon avidus*. Herein, based on the mitochondrial DNA (mtDNA) sequences of the cytochrome *c* oxidase subunit I (COI) and morphometric wing parameters, *M. avidus* is shown to comprise a complex of cryptic species, and one variety is redefined as a valid species: *M. bicolor* Gil Collado, 1930 (as var. of *spinipes*). The species *M. bicolor, M. avidus* A, and *M. avidus* B were clearly delimited based on their wing size. A total of 29 *M. avidus* and *M. bicolor* individuals presented 20 mtDNA haplotypes, four of which were shared by *M. avidus* A and *M. avidus* B, three were confined to *M. bicolor*, seven to *M. avidus* A, and six to *M. avidus* B. Sequence divergences between lineages occurring in the Balkan and in Spain ranged from 4.93 to 6.0 (uncorrected *p* in %) whereas divergences between *M. avidus* A and *M. avidus* B were 0.26 to 1.56. Divergence among morphologically identified individuals of *M. avidus* A and *M. avidus* B species ranged from 0.13 to 1.58, and from 0.13 to 0.52, respectively. The phenotypic substructuring and observed genetic uniqueness of populations in spatially and temporally fragmented *M. avidus* taxa were used to identify genetic units. The early split of two allopatric lineages, Spanish *M. bicolor* and Balkan *M. avidus*, was followed by diversification in each lineage. Present-day morphological uniformity masks much of the genetic complexity of lineages within the *M. avidus* complex. © 2009 The Linnean Society of London, *Zoological Journal of the Linnean Society*, 2009, **155**, 819–833.

ADDITIONAL KEYWORDS: COI mtDNA – cryptic species – geometric morphometrics – interspecific variation – intraspecific variation.

INTRODUCTION

Delimitation of biological diversity is a crucial step in understanding phenomena in evolutionary biology, ecology, and biogeography. Cryptic species are a group of reproductively isolated but morphologically inseparable species, and are often classified as a single nominal species (cf. Bickford *et al.*, 2006). Investigation of cryptic speciation provides new insights into genetic and species (hidden) diversity (e.g. Schmitt *et al.*, 2006), and the occurrence of cryptic species has conservation implications as well (Finston *et al.*, 2007; Smith & Friesen, 2007).

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European biota experienced dramatic climatic oscillations during the Pleistocene. As a result of repeated cycles of contractions and expansions of species' ranges, isolation in refugia and (re)colonizations, genetic divergence with speciation, and secondary contacts of previously distinct populations (Taberlet *et al.*, 1998), the Balkan and Iberian Peninsulas became sources of large species diversity (Hewitt, 2000, 2004). Indeed, the phylogeographic structure and distribution of genetic variation of species reflect the complex biogeographic history of those regions (e.g. Pincell *et al.*, 2005). One such example is the genus *Merodon* Meigen, 1803, a diverse European hoverfly taxon, with centres of endemism and diversity on the Balkan and Iberian Peninsulas

(Marcos-García, Vujic & Mengual, 2007). Assessing taxonomic relationships within the genus *Merodon* has been a challenge because of the presence of multiple cryptic taxa, and the lack of consistent and reliable diagnostic markers. Although some cryptic taxa of hoverflies have been delineated using molecular markers (cf. Milankov *et al.*, 2008), mitochondrial DNA (mtDNA) sequence variability and the rate of evolution are inconsistent within and among species. No standard level of divergence can be used to establish species boundaries and no standardized distance can be applied for all species (e.g. Lipscomb, Platnick & Wheeler, 2003; Will & Rubinoff, 2004).

Recently, the use of geometric morphometrics has helped to resolve taxonomic problems (e.g. Moraes *et al.*, 2004) and quantify phenotypic variability in some insect groups (Hoffmann & Shirriffs, 2002; Gumiel *et al.*, 2003). While both wing size and shape show some evidence of adaptation (e.g. Gilchrist *et al.*, 2000; Kölliker-Ott, Blows & Hoffmann, 2003), wing size and shape have been shown to have different genetic properties, with size heritability being generally low, whereas wing shape is less sensitive to environmental changes and is highly heritable (Bitner-Mathé & Klaczko, 1999; Birdsall *et al.*, 2000).

In this study, mtDNA diversity analysis was used in conjunction with morphometric wing parameters to determine evolutionarily independent genetic units within the morphologically defined *Merodon avidus* (Rossi, 1790) species. Ever since its first description, the species *M. avidus* has been the subject of taxonomic debate because of great variation in the coloration of antennae, thorax, legs, and abdomen, resulting in 24 known synonyms (Hurkmans, 1993). Today we know that the widespread species *M. avidus* is actually a geographically and genetically structured taxon comprising a diverse group of cryptic taxa (Milankov, Vujić & Ludoški, 2001). In a recent study of allozyme variability in populations of *M. avidus*, two cryptic species designated as *Merodon avidus* A (Mediterranean region and central part of the Balkan Peninsula) and *Merodon avidus* B (mountainous regions of the Balkan Peninsula), were identified based on the diagnostic species-specific alleles at the *Idh-2* and *Aat* loci (Milankov *et al.*, 2001). Morphological analysis of tergite II and III, tibiae, and mesoscutum confirmed the existence of at least two taxa (Milankov *et al.*, 2001).

The goal of this study was to assess phenotypic variation in wing shape for sympatric and allopatric taxonomic units, and to determine whether the pattern of variation was consistent with character displacement. Furthermore, the utility of wing size and shape for detecting levels of intraspecific variation in hoverflies was examined by assessing phenotypic and genotypic variations across spatially and temporally fragmented populations. The results reported herein provide an insight into phenotypic and genetic diversity of the *M. avidus* taxa and provide a basis for forthcoming studies of this group.

MATERIAL AND METHODS

Sample collection

Samples of 11 populations of the *M. avidus* taxa were collected over several years from eight regions (Fig. 1) on the Balkan Peninsula and in Spain (population code and number of collected specimens are given in Table 1). Specimens were initially identified as members of the *M. avidus* A and *M. avidus* B species based on the species-specific alleles and their combination at diagnostic allozyme *Idh-2* and *Aat* loci as well as morphological taxonomic traits (Milankov *et al.*, 2001). In addition, type material of species from the *M. avidus* complex from Spain was also studied. To date, the only name published from this complex is *Merodon spinipes* var. *bicolor* Gil Collado, 1930, described from an unspecified number of male and female specimens. One type specimen (syntype) is deposited at the Madrid Museum (MNCN-Museo Nacional de Ciencias Naturales, Madrid, Spain).

Tentative groups of spatially separated and closed populations were recognized: populations from Greece and Former Yugoslav Republic of Macedonia were considered metapopulations, five Pannonian populations (Serbia) were pooled together (designated as APAN below), whereas specimens from both Dubašnica Mt (Serbia) and Durmitor Mt (Montenegro) were taken from several biogeographically different sites and constituted a metapopulation (Table 1). Groups of populations were analysed for the existence and level of population substructuring.

Morphometric analysis

Geometric morphometric analysis included specimens of *M. avidus* A ($n = 269$), and *M. avidus* B ($n = 230$) from 27 localities in the Balkan Peninsula, and two specimens from Spain initially identified as *M. avidus* B (Table 1, Fig. 1). The right wing of all flies was removed and mounted in Hoyer's medium between microscope slides. Wing images were captured using a digital camera (Leica DFC320) connected to a stereomicroscope (Leica MZ12.5). Ten landmarks positioned at vein intersections or terminations were collected using TpsDig 1.40 and expressed as *x,y* coordinates in a Cartesian space (Rohlf, 2004; Fig. 2).

Wing size variation was examined using centroid size (the square root of the sum of squared distance between each landmark and the wing centroid), an isometric estimator of size. One-way analysis of vari-

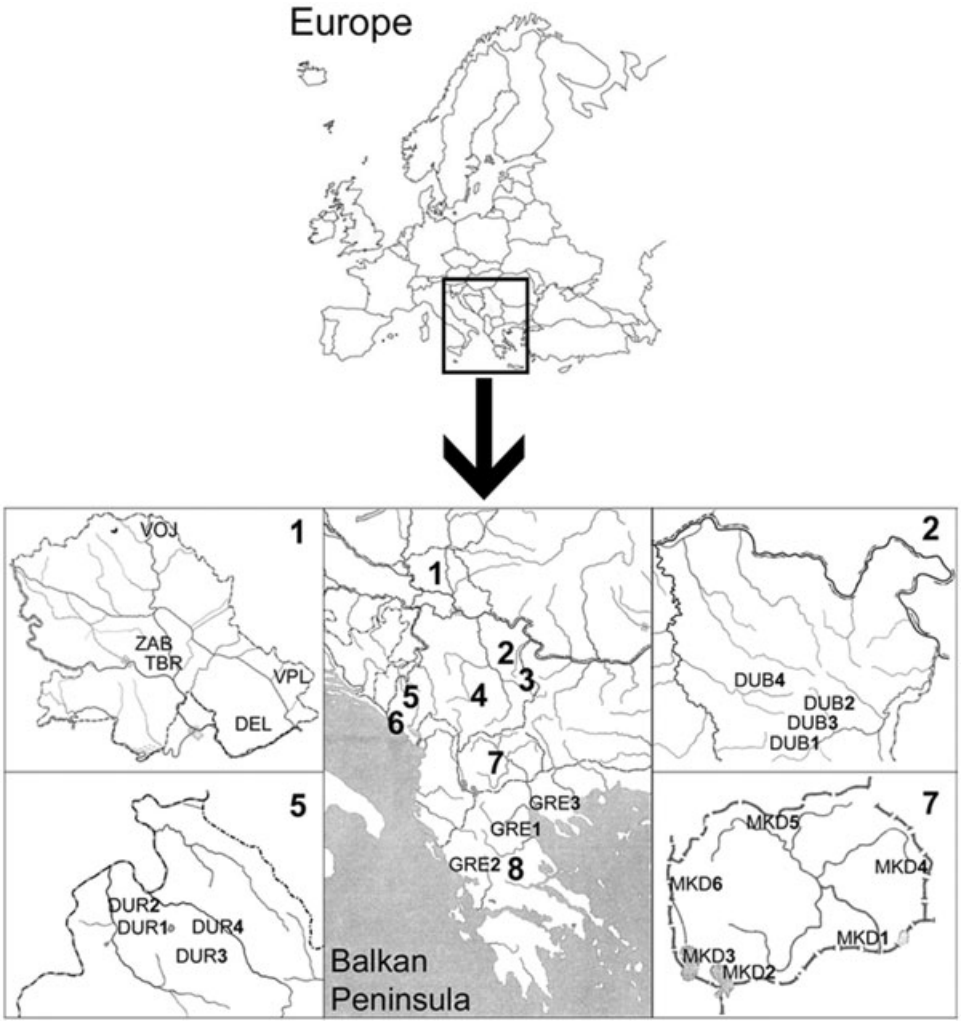

Figure 1. Origin of the analysed populations using wing geometric morphometrics from the Balkan Peninsula: 1, Pannonian Plain (PAN, Serbia); 2, Dubašnica Mt, E 21°59′, N 44°01′ (DUB, Serbia); 3, Stara Mt, E 22°41′, N 43°20′ (SPL, Serbia); 4, Kopaonik Mt, E 20°40′, N 43°15′ (KOP, Serbia); 5, Durmitor Mt, E 19°00′, N 43°11′ (DUR, Montenegro); 6, Morinj, E 18°40′, N 43°29′30″ (MOR, Montenegro); 7, Former Yugoslav Republic of Macedonia (MKD); 8, Greece (GRE). (Numbers within region are noted sampling sites of populations).

ance (ANOVA) was used to test differences in centroid size among species, populations, and between sexes.

For the wing shape analysis, landmarks of each specimen were first aligned using a generalized Procrustean analysis procedure to remove the nonshape effects of translation, rotation, and scale (Rohlf & Slice, 1990), and then a thin-plate spline analysis was carried out. The resulting matrix (*w*; 'weight matrix' of Rohlf, Loy & Corti, 1996) was used for canonical variate analysis (CVA) to examine the pattern of within-species/population variation in total shape space. Species (used as a group variable in CVA) were a priori defined based on conventional morphologic characters (defined in Milankov *et al.*, 2001) and allozymes (if specimens were included in the allozyme variability study conducted by Milankov *et al.*, 2001). Differences in wing shape and size were analysed by comparing phenotypic traits of female and male specimens separately. As the interspecific analysis of female wing shape showed significant differences, allopatric and sympatric populations were compared (because of the absence of female specimens, *M. avidus* B KOP and *M. avidus* B MKD population samples were omitted from this analysis).

Procrustean superimpositions, calculation of the centroid size and *w* matrix were performed using TpsRelw 1.44 (Rohlf, 2006) and multiple regressions and visualization of deformation grids were computed using TpsRegr 1.31 (Rohlf, 2005). All programs for collection of wing landmarks and geometric

Table 1. The *Merodon avidus* group: species, sampling sites, and codes for metapopulations and populations, and sample size for morphometric analysis

	Metapopulation		Population				
Species	Sampling site	Code	Sampling site	Code	♂	♀	Σ
M. avidus A	Pannonian Plain	APAN	Titelski breg	ATBR	24	5	54
			Filić	AVOJ	5	2	
			Vršačke Mt	AVPL	8	2	
			Žabalj	AZAB	4	1	
			Deliblato sand	ADEL	2	1	
	Dubašnica Mt	ADUB	Malinik Mt	ADUB1	6	–	43
			Dubašnica Mt	ADUB2	2	–	
			Lazareva reka gorge	ADUB3	29	6	
			Morinj	AMOR	69	20	89
	MKD	AMKD*	Kožuf Mt	AMKD1	11	3	49
			Baba Mt	AMKD2	4	1	
			Oteševo	AMKD3	12	5	
			Berevo	AMKD4	6	2	
			Skopje	AMKD5	5	–	
	Greece	AGRE	Olimp Mt	AGRE1	8	3	34
			Pindos Mt	AGRE2	9	5	
			Halkidiki	AGRE3	8	1	
M. avidus B	Dubašnica Mt	BDUB	Malinik Mt	BDUB1	10	–	64
			Dubašnica Mt	BDUB2	7	–	
			Lazareva reka gorge	BDUB3	36	6	
			Beljanica Mt	BDUB4	5	–	
			Kopaonik Mt	BKOP	16	1	17
			Stara Mt	BSPL	26	13	39
	Durmitor Mt	BDUR	Sušičko lake	BDUR1	5	1	67
			Sušica gorge	BDUR2	7	4	
			Žabljak plain	BDUR3	35	13	
			Tara gorge	BDUR4	1	1	
	MKD	BMKD	Mavrovo	BMKD6	5	1	6
	Greece	BGRE	Olimp Mt	BGRE1	2	–	37
			Pindos Mt	BGRE2	27	8	
M. bicolor	Spain		Ciudad Real		2	–	2

*MKD is the official three-letter code for the Former Yugoslav Republic of Macedonia (FYR MACEDONIA).

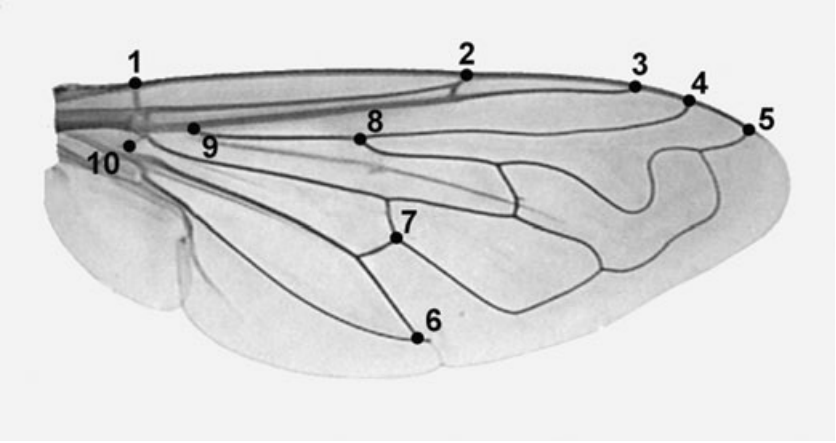

Figure 2. Positions of the wing landmarks used in the geometric morphometric analyses.

Table 2. DNA voucher no., Locality, GenBank accession no. and location of DNA voucher specimen (FMNH, Finland) of *Merodon avidus* A and *Merodon avidus* B *sensu* Milankov *et al.*, 2001

DNA voucher no.	Taxon	Population code	Locality and date	COI
VM566	*Merodon avidus* A	AMOR	Montenegro, Morinj 18.06.1998	DQ845109
VM567	*Merodon avidus* A	AMOR	Montenegro, Morinj 18.06.1998	DQ845110
VM581	*Merodon avidus* A	AMOR	Montenegro, Morinj 31.08.1998	DQ845111
VM596	*Merodon avidus* A	AMOR	Montenegro, Morinj 31.08.1998	DQ845112
VM579	*Merodon avidus* A	AMOR	Montenegro, Morinj 25.04.1998	DQ845113
VM580	*Merodon avidus* A	AMOR	Montenegro, Morinj 25.04.1998	DQ845114
VM615	*Merodon avidus* A	ADUB	Serbia, Dubašnica Mt 19.09.1997	DQ845115
VM616	*Merodon avidus* A	AMOR	Serbia, Dubašnica Mt 19.09.1997	DQ845116
VM578	*Merodon avidus* A	APIN	Greece, Pindos Mt 20.05.1997	DQ845117
VM561	*Merodon avidus* A	APIN	Greece, Pindos Mt 20.05.1997	DQ845118
VM590	*Merodon avidus* B	BDUR	Montenegro, Durmitor Mt 25.06.1997	DQ845119
VM589	*Merodon avidus* B	BPIN	Montenegro, Durmitor Mt 20.06.1998	DQ845120
VM605	*Merodon avidus* B	BDUR	Montenegro, Durmitor Mt 20.06.1998	DQ845121
VM560	*Merodon avidus* B	BMAV	FYR Macedonia, Mavrovo lake 10.07.1998	DQ845122
VM572	*Merodon avidus* B	BMAV	FYR Macedonia, Mavrovo lake 10.07.1998	DQ845123
VM591	*Merodon avidus* B	BPIN	Greece, Pindos Mt 15.07.1998	DQ845124
VM563	*Merodon avidus* B	BPIN	Greece, Pindos Mt 15.07.1998	DQ845125
VM571	*Merodon avidus* B	BDUB	Serbia, Dubašnica Mt 21.08.1997	DQ845126
VM557	*Merodon avidus* B	BDUB	Serbia, Dubašnica Mt 03.06.1996	DQ845127
VM558	*Merodon avidus* B	BDUB	Serbia, Dubašnica Mt 08.06.1997	DQ845128
VM583	*Merodon avidus* B	BDUB	Serbia, Dubašnica Mt 01.07.1998	DQ845129
VM823	*Merodon avidus* A	ALES	Greece, Lesvos, Sikaminia, 24.04.2001	DQ845130
VM824	*Merodon avidus* A	ALES	Greece, Lesvos, Sikaminia, 24.04.2001	DQ845131
S409	*Merodon avidus* A	ALES	Greece, Lesvos, Vatoussa, 20-28.IV.2001	DQ845132
S524	*Merodon avidus* B	BFRA	France, 10 km S, 18.05.2003	DQ845133
S532	*Merodon avidus* A	ALES	Greece, Lesvos, Plomari, 14.07.2004	DQ845134
VM826	*Merodon bicolor* Gil Collado 1930		Spain, PN de Cabaneros, Ciudad Real 05.07.2005	DQ845135
VM827	*Merodon bicolor* Gil Collado 1930		Spain, PN de Cabaneros, Ciudad Real 11.06.2005	DQ845136

morphometric calculations are freeware available at http://life.bio.sunysb.edu/morph/. ANOVA and CVA were completed using STATISTICA for Windows (version 7.1).

DNA SEQUENCING

Molecular analysis of taxa of the *M. avidus* group included specimens from Spain, France, and the Balkan Peninsula (Table 2), although specimens from Lezvos (Greece) and France were not available for wing geometric morphometric analysis. 768- or 520-bp fragments of the cytochrome *c* oxidase subunit I (COI) gene were sequenced from 29 individuals. DNA was extracted from legs or other parts of the fly remaining after allozyme electrophoresis (Milankov *et al.*, 2001) using the Nucleospin Tissue DNA extraction kit (Machery-Nagel, Düren, Germany) following the manufacturer's protocols and then re-suspended in 50 µL ultra-pure water. The obtained DNA fragments corresponded to nucleotide positions 2233 to 3000 and 2752 in *Drosophila yakuba* sequence (Clary & Wolstenholme, 1985). The sequences were deposited in GenBank (accession numbers of the analysed specimens are listed in Table 2). Remains of specimens, including male genitalia, used for the morphological studies and for DNA extraction were deposited at the Finnish Museum of Natural History (Helsinki, Finland) and Department of Biology and Ecology, Faculty of Sciences (University of Novi Sad, Serbia).

PCR reactions were carried out in 25 µL reaction aliquots containing 2 µL DNA extract, 1 µL of each primer (at 10 pmol μL^{-1}), 0.25 µL DNA polymerase (5 U μl^{-1}), 2 µL 2.5 mM $MgCl_2$, 2.5 µL 10X Buffer II (MBI Fermentas, St. Leon-Rot, Germany), 4 µL 200 mM dNTP (GeneAmp, Applied Biosystems, Foster City, CA, USA), and ultra-pure water. Thermocycler conditions were initial denaturing at 95 °C 2 min, 29 cycles of 30 s denaturing at 94 °C, 30 s annealing at 49 °C, 2 min extension at 72 °C, followed by a final extension of 8 min at 72 °C. The universally conserved primers used for amplifying and sequenc-

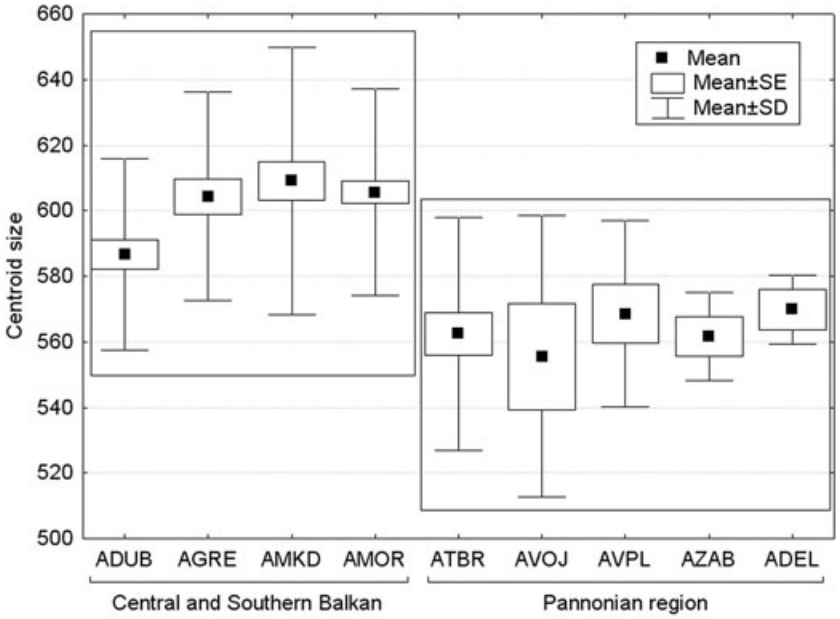

Figure 3. Box plot of centroid size for nine populations of *Merodon avidus* A species. The mean centroid size of populations from the central and southern Balkans is larger than that for populations from the Pannonian region ($F_{(1,267)} = 58.57$, $P < 0.001$).

ing the COI fragment (768 bp J+P; 520 bp J+I) were the forward primer C1-J-2183 (5′-CAA CAT TTA TTT TGA TTT TTT GG-3′) (alias JERRY) and two reverse primers TL2-N-3014 (5′-TCC AAT GCA CTA ATC TGC CAT ATT A-3′) (alias PAT) (Simon *et al.*, 1994) and C1-N-2735 (5′-AAA ATG TTG AGG GAA AAA ATG TTA-3′) (alias INGER) (Lunt *et al.*, 1996). PCR products were purified using the GFX PCR Purification Kit (GE Healthcare Biosciences, Little Chalfont, UK) and then sequenced (with the PCR primers) in both directions using the Big Dye Terminator Cycle Sequencing Kit (version 1.1, Applied Biosystems) at one quarter of the recommended volumes on an ABI PRISM 377 (Applied Biosystems) semi-automated DNA sequencer. The sequences were edited for base-calling errors and assembled using Sequence Navigator (version 1.01, Applied Biosystems).

Phylogenetic analyses

Alignment of COI sequences was trivial because of the lack of indels and was carried out by eye. Nucleotide divergences within and among species were calculated using uncorrected *p* distances. Parsimony analysis used 29 ingroup terminals and was performed using NONA (Goloboff, 1999) and spawned with the aid of WINCLADA (Nixon, 2002), using a heuristic search algorithm with 1000 random addition replicates (mult*1000), holding 100 trees per round (hold/100), maxtrees set to 100 000, and applying tree bisection-reconnection (TBR) branch swapping. All base positions were treated as equally weighted characters. *Merodon testaceus* Sack, 1913 was used as the outgroup, and *Merodon elegans* Hurkmans, 1993 was included as well (GenBank accession numbers are EF591084 and DQ386328, respectively).

RESULTS

Phenotypic diversity

The analysis of variance of wing centroid size revealed significant differences between *M. avidus* A and *M. avidus* B species ($F_{(1,497)} = 130.34$, $P < 0.001$), and between male ($F_{(1,392)} = 103.91$, $P < 0.0001$) and female individuals ($F_{(1,95)} = 34.42$, $P < 0.0001$) of each species considered separately. Centroid size was a highly significant factor among *M. avidus* A populations ($F_{(8,260)} = 9.15$, $P < 0.001$), allowing discrimination between populations from the Pannonian region (smaller wing size) and populations originating from central and southern parts of the Balkan Peninsula (larger wing size; Fig. 3). Similarly, centroid size variation among the six conspecific populations of *M. avidus* B was significant ($F_{(5,224)} = 2.83$, $P = 0.017$).

Sexual size dimorphism (SSD) in wing size but not in wing shape was observed. Wings were considerably larger in female than in male specimens in both *M. avidus* A and *M. avidus* B species ($F_{(1,267)} = 28.86$, $P < 0.001$; $F_{(1,228)} = 16.14$, $P = 0.001$, respectively; Fig. 4). Only in the *M. avidus* A from Dubašnica Mt population was no difference in wing size between

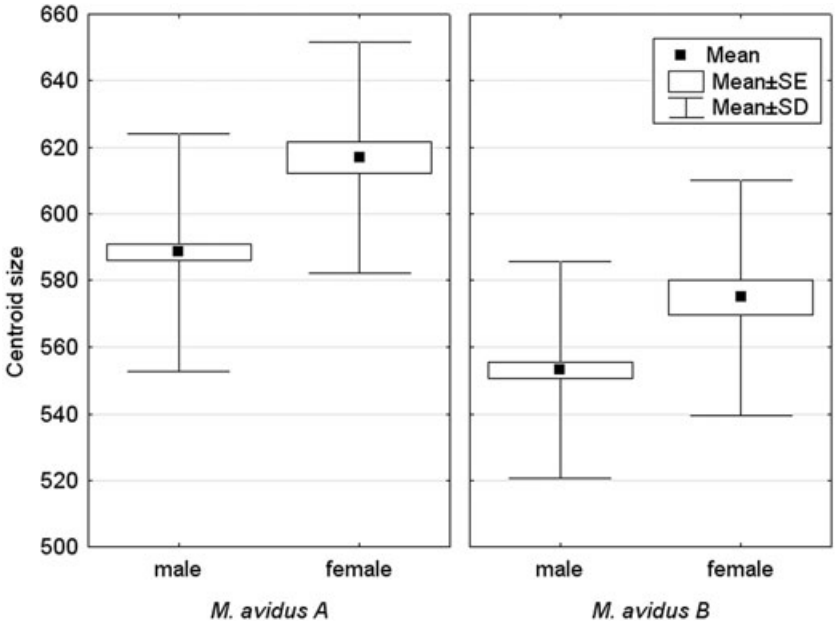

Figure 4. Wing centroid size in male and female *Merodon avidus* A and *M. avidus* B.

male and female individuals detected. Intraspecific variation in wing size calculated separately for each sex revealed significant differences among male populations of both *M. avidus* A ($F_{(4,207)} = 16.52$, $P < 0.001$) and *M. avidus* B ($F_{(5,176)} = 4.27$, $P = 0.001$). Wing size was less variable among female populations, with no significant differences within *M. avidus* B ($F_{(3,42)} = 0.81$, $P = 0.49$) and significant variability in populations of *M. avidus* A ($F_{(4,52)} = 3.88$, $P = 0.008$). Intraspecific within-sex comparison of populations did not detect any correlations between wing size and latitude of locality. There were no significant differences in wing size within analysed metapopulations.

CVA with species as a grouping variable performed on the *w* matrix was significant when sexes were considered separately (Wilks' $\Lambda = 0.43$; $F_{(48,1428)} = 9.86$; $P < 0.0001$). CVA on the *w* matrix with populations as a grouping variable for each species indicated a good separation of populations within a metapopulation, with the range of correct classification between 83 and 98% (Fig. 5). The thin-plate spline visualizations showed that most of the shape changes were associated with differences in the relative positions of landmarks 6, 7, 8, and 2, which influenced the wing's width and length (not shown).

Wings of female specimens of sympatric metapopulations of *M. avidus* A were significantly larger than those of *M. avidus* B from Greece ($F_{(1,15)} = 15.57$, $P = 0.001$) and could be clearly separated based on wing shape as well (Fig. 6A). Female specimens from sympatric populations from Dubašnica Mt differed in wing shape (Fig. 6A), but not in wing size ($F_{(1,10)} = 0.07$, $P = 0.802$). The associated deformation grids suggest that the shape differences are a result of displacement of landmarks 7 and 8 (not shown).

Overall, there was a great deal of similarity between allopatric metapopulation pairs of *M. avidus* A and *M. avidus* B species in wing shape (Fig. 6B–D) and wing size (*M. avidus* A from MKD/Morinj: $F_{(1,29)} = 0.89$, $P = 0.35$; *M. avidus* B from Durmitor Mt/Stara Mt: $F_{(1,30)} = 0.004$, $P = 0.95$) even though some metapopulations did differ in size (*M. avidus* A from Pannonian region/MKD: $F_{(1,20)} = 9.46$, $P = 0.006$; *M. avidus* A from Pannonian region/Morinj: $F_{(1,29)} = 8.58$, $P = 0.007$). Based on wing shape, a distinct population of *M. avidus* A from Morinj was noted (Fig. 6B, D), with reduced distances between wing landmarks 6 and 2, and 8 and 9 (not shown).

COI VARIATION AND PARSIMONY ANALYSIS

A total of 20 haplotypes, defined by 53 variable positions, was found in 29 analysed specimens (Fig. 7). The lack of identical individuals within one population of nominated species indicated extensive intraspecific polymorphism. Seven unique haplotypes corresponded to *M. avidus* A and six to *M. avidus* B, whereas four were shared by *M. avidus* A and *M. avidus* B (Table 3; Fig. 7).

Haplotypes formed two main clades corresponding to *M. bicolor* from Spain, and *M. avidus* A + B from the Balkan and French clade that differed by 29 or more nucleotides. Within the Spanish haplotypes

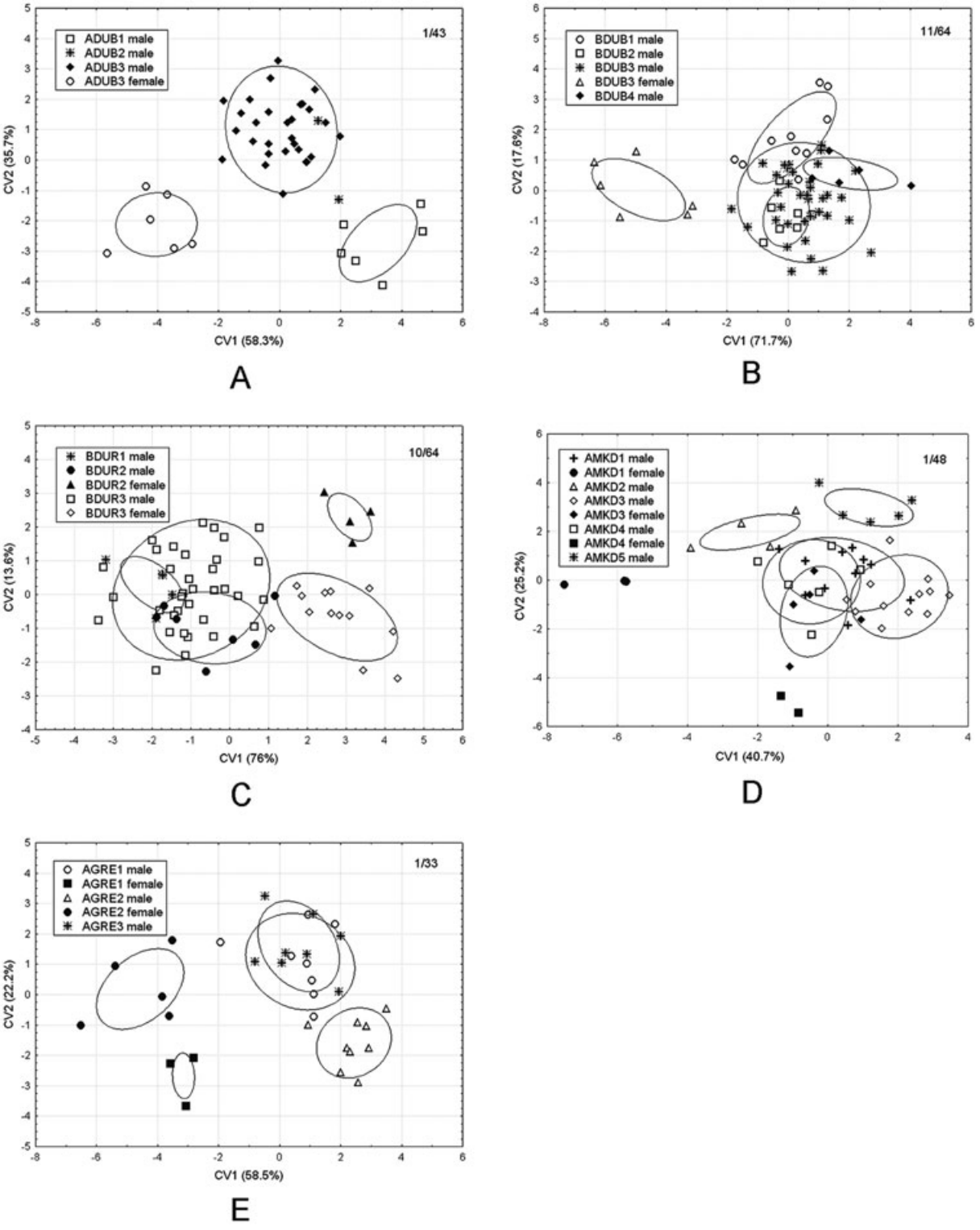

Figure 5. Scatterplot of individual scores from the canonical variate analysis (CVA) of all specimens (both sexes) of metapopulations: A, *Merodon avidus* A from Dubašnica Mt (ADUB) (Wilks' $\Lambda = 0.04$; $F_{(48,72)} = 3.06$; $P < 0.000$); B, *M. avidus* B from Dubašnica Mt (BDUB) (Wilks' $\Lambda = 0.10$; $F_{(64,174)} = 2.18$; $P < 0.000$); C, *M. avidus* B from Durmitor Mt (BDUR) (Wilks' $\Lambda = 0.10$; $F_{(64,174)} = 2.21$; $P < 0.000$); D, *M. avidus* A from FYR MACEDONIA (AMKD) (Wilks' $\Lambda = 0.002$; $F_{(112,171)} = 2.40$; $P < 0.000$); E, *M. avidus* A from Greece (AGRE) (Wilks' $\Lambda = 0.009$; $F_{(64,53)} = 1.93$; $P < 0.007$). The amount of variation explained by each canonical axis is in parentheses. The number of misclassified specimens/total number of analysed specimens is shown in each panel.

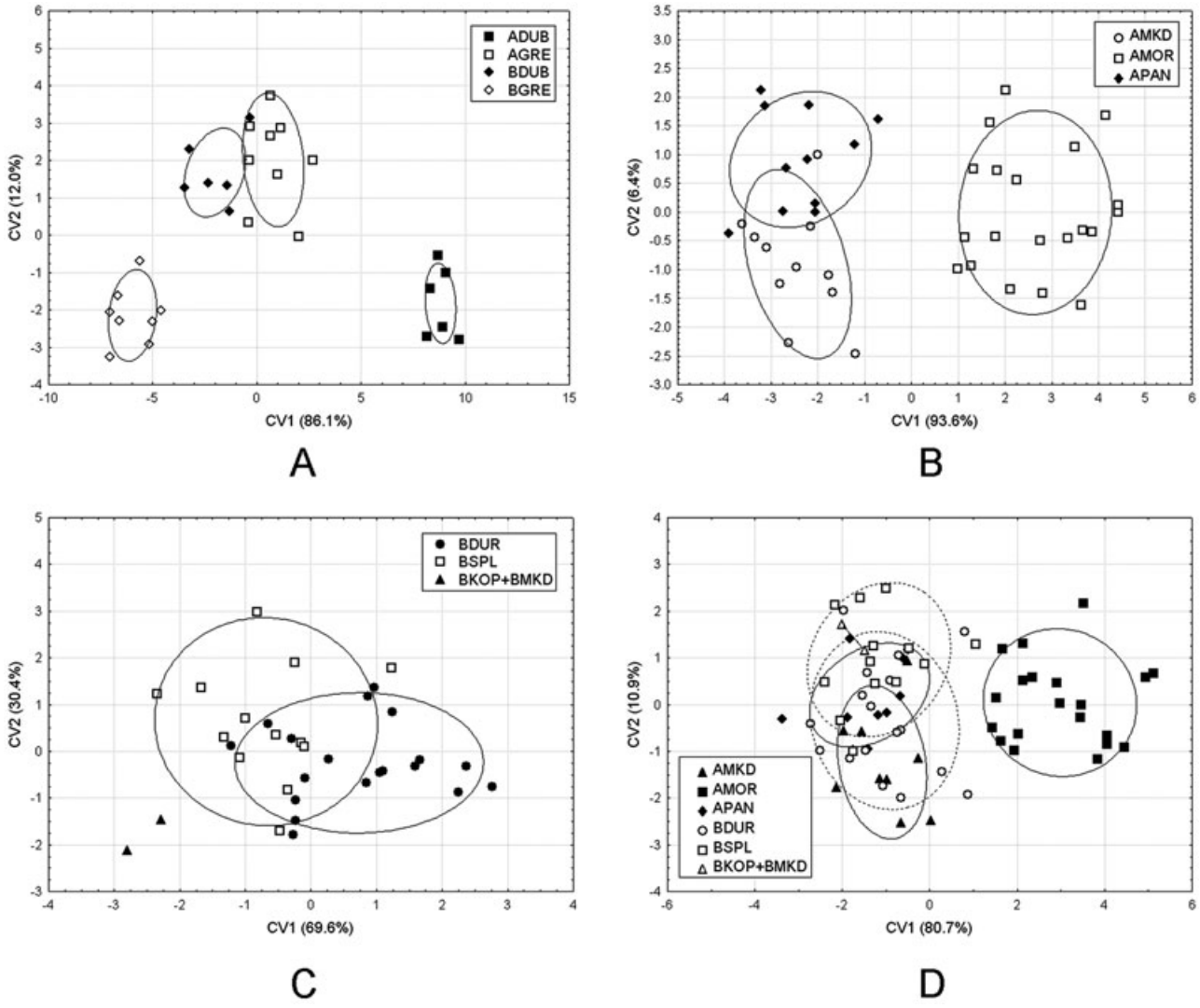

Figure 6. Scatterplot of individual scores from the canonical variate analysis (CVA) of female specimens of: A, sympatric populations of *Merodon avidus* A and *M. avidus* B from Dubašnica Mt (ADUB, BDUB) and Greece (AGRE, BGRE) (Wilks' $\Lambda = 0.003$; $F_{(48,30)} = 3.64$; $P < 0.001$); B, allopatric populations of *M. avidus* A from FYR MACEDONIA (AMKD), Morinj (AMOR), and the Pannonian region (APAN) (Wilks' $\Lambda = 0.09$; $F_{(32,48)} = 3.59$; $P < 0.0001$); C, allopatric populations of *M. avidus* B from Durmitor Mt (BDUR), Stara Mt (BSPL), Kopaonik Mt (BKOP), and FYR MACEDONIA (BMKD) (Wilks' $\Lambda = 0.36$; $F_{(32,32)} = 0.66$; $P < 0.879$); D, allopatric populations of *M. avidus* A and *M. avidus* B (Wilks' $\Lambda = 0.09$; $F_{(80,245)} = 1.94$; $P < 0.0001$). The amount of variation explained by each canonical axis is in parentheses.

there were one or two nucleotide changes. Within *M. avidus* A + B from the Balkan and French clade, there were low nucleotide differences among I, II, III vs. VII, VIII; IV vs. XIV, XV; V vs. XIV; VII vs. VIII, XVI; VIII vs. XIV; and XIV vs. IX, X, XI (Figs 7, 8).

Sequence divergences (uncorrected *p* divergences in %) between the Balkan and Spanish clades ranged from 4.93 to 6.0, whereas divergences between *M. avidus* A and *M. avidus* B were 0.26 to 1.56. Sequence divergences within populations (0.26–1.43) were similar to the range of divergences among conspecific populations of *M. avidus* A (0.13–1.58), whereas lower divergences among conspecific populations were observed for *M. avidus* B (0.13–0.52; Table 4).

The parsimony analysis of 29 ingroup terminals of the *M. avidus* complex using *M. testaceus* as the outgroup resulted in > 200 equally parsimonious trees with a length of 116 steps (consistency index = 0.81, retention index = 0.87; Fig. 8).

MERODON BICOLOR GIL COLLADO, 1930 **COMB. NOV.**

Merodon spinipes bicolor Gil Collado, 1930: 254

Based on morphology (Marcos-García *et al.*, 2007), *M. avidus* from Spain (Marcos-García, 1985, 1990)

	Population	Haplotype	2222222222222222222222222222222222222222222222222222 2223333334444444444555555566666666677777778888888999 5591446890113355578900134580002245591245679002377956 9 3659061216253714728025724934782892855457986286517324 8
			* * * * *
VM567	AMOR, June	I	TATATCCTTTTAATCTTTGTACATTCCTATTTTCTCCCCCAATTTTCTCTTGC
VM581	AMOR, August	II	.....C....T...G.....C.....
VM596*	AMOR, August	III	.G.....C....T.----------
VM615	ADUB, September	IV	.....T..G.....C...A.
VM590	BDUR	IV	.....T..G.....C...A.
VM823	*M. avidus*, Lesvos	IV	.....T..G.....C...A.
VM560	BMAV	V	.....T..GG.....C.....
VM572*	BMAV	IV/V	.....T.----------
VM578*	APIN, May	IV/V	.....T.----------
VM591*	BPIN, July	IV/V	.....T.----------
VM824	*M. avidus* A, Lesvos	VI	.....A.
VM561	APIN, May	VII	.....C....T.....C.....
VM616	ADUB, September	VII	.....C....T.....C.....
VM571	BDUB, August	VII	.....C....T.....C....-
VM557	BDUB, June	VIII	.....C....T.....-
S409	*M. avidus* A, Lesvos	VIII	.....C....T.....
VM589	BDUR, June	IX	.....A.....T..G.....C.....
VM605	BDUR, June	X	.....CC.....T..G.....C.....
VM563	BPIN, July	XI	.....T..G.....CT....
VM566	AMOR, June	XII	.....T.....C.T.....T.....C..TC.C.A.
VM558	BDUB, June	XIII	.....C....T.....C.....
VM583	BDUB, July	XIV	.....T..G.....C....-
VM579	AMOR, April	XIV	.....T..G.....C.....
S524	*M. avidus* B, France	XV	.....T.....C...A.
VM580	AMOR, April	XVI	.....C.C..T.....C.....
S532	*M. avidus* A, Lesvos	XVII	C.C.....C.....C.C.....T.....T.....CC..C.C.A.
X14**	*M. bicolor,* Spain	XVIII	..CTCCTCCC.GCCTAA...TTT.CTTC.CCCCTCTTTTTCTCCCCTC.CCAT
VM826	*M. bicolor,* Spain	XIX	..CTCCTCCC.GCCTAA...TTT.CTTC.CCCCT.TTTTTCTCCCCTC.CCAT
VM827	*M. bicolour,* Spain	XX	..CTCCTCCC GCCTAA...TTT.CTTCGCCCCTCTTTTTCTCCCCTC.CCAT

Figure 7. Mitochondrial cytochrome *c* oxidase subunit I (COI) haplotype variation of 53 noncontinuous sites obtained from the *Merodon avidus* taxa (DUB, Dubašnica Mt; MOR, Morinj; PIN, Pindos Mt; LES, Lesvos; DUR, Durmitor Mt; MAV, Mavrovo Lake). * synonymous substitution at first codon position; *sequences' length 520 bp; **the specimen X14 was published under the name *M. avidus* B in Mengual *et al.* (2006).

was initially identified as *M. avidus* B *sensu* Milankov *et al.* (2001) (Mengual *et al.*, 2006; Marcos-García *et al.*, 2007).

We propose the name *M. bicolor* Gil Collado, 1930: 254 (*Merodon*, as var. of *spinipes*) (identity: valid species: comb. nov.) for the cryptic taxon from the Iberian Peninsula. *Merodon bicolor* was described from the three syntypes (Gil Collado, 1930), as a variety of *M. spinipes* (Fabricius) from following localities: El Escorial, Cazzurro, Cercedilla, Arias, Somosiera, G. Menor. In depositary museum (Instituto Español de Entomología, Madrid, Spain), only one syntype was found. Based on this type specimen, the lectotype of this taxon was designated here: male '*spinipes* v. *bicolor* / Cazurro' (El Escorial, Spain) (MNCN). The lectotype shared the morphological characters with specimens from Spain analysed in this paper (Tables 1, 2).

Wing size ($F_{(2,498)} = 65.57$, $P < 0.001$) and shape (Wilks' $\Lambda = 0.49$; $F_{(32,966)} = 12.75$; $P < 0.000$) of two the available specimens from Spain were distinctly different from both *M. avidus* A and *M. avidus* B from the Balkan Peninsula. COI mtDNA sequence divergences between the Balkan and Spanish clades ranged from 4.93 to 6.0 (Table 4). Thus, wing morphometrics and COI mtDNA haplotypes allowed clear delineation of these cryptic taxa.

DISCUSSION

Phenotypic and molecular diversity

Significant morphological divergence among female specimens in each sympatric pair of *M. avidus* A and *M. avidus* B, and a substantial overlap in shape variability in allopatric populations, suggested that the divergence in wing shape in co-occurring taxa might have been generated by a balance of ecological and reproductive character displacement. We also observed that female specimens had generally larger

Table 3. Geographic distribution of haplotypes in taxa of the *Merodon avidus* group

Taxon	Haplotype																			
	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	XIII	XIV	XV	XVI	XVII	XVIII	XIX	XX
M. avidus A																				
ADUB				**x**			**x**													
AMOR	x	x	x									x		**x**		x				
APIN							**x**													
ALES				**x**		x		**x**									x			
M. avidus B																				
BDUB							**x**	**x**					x	**x**						
BDUR				**x**					x	x										
BMAV					x															
BPIN											x									
BFRA															x					
M. bicolor																		x	x	x

Common haplotypes of *M. avidus* A and *M. avidus* B in bold.
DUB, Dubašnica Mt; MOR, Morinj; PIN, Pindos Mt; LES, Lesvos; DUR, Durmitor Mt; MAV, Mavrovo Lake; FRA, France.

wings than male specimens of the analysed *Merodon* taxa, which is in accordance with about 80% of investigated insect species (Teder & Tammaru, 2005). It is possible that character displacement resulted from competition for limited resources (Schluter, 2000), while difficulties for sympatric species in identifying conspecific mates could have led to reproductive character displacement (cf. Rice & Pfenning, 2006). However, it is difficult to determine which form of character displacement, either ecological or reproductive, might have been a predominant factor in wing shape divergence of the focal taxa because little is known about their specific ecological and mating preferences. To date, territorial behaviour has been registered only for male *M. avidus* (Speight, 2007). As aerodynamic performance and courtship song in dipteran species are likely to be affected by wing shape (Birdsall *et al.*, 2000), we hypothesize that significant morphological differentiation among sympatric populations might have been caused by reproductive character displacement.

In the present study, mtDNA sequencing revealed extensive haplotype variation in the *M. avidus* group. However, mtDNA COI markers failed to discriminate evolutionarily independent sympatric genetic units, previously identified as *M. avidus* A and *M. avidus* B, using fixed allozyme differences at the diagnostic loci (Milankov *et al.*, 2001) and delimited based on wing morphometrics (see above). The importance of integrating molecular and morphological characters (e.g. Rubinoff & Holland, 2005; Rubinoff, 2006), especially when dealing with closely related species, recently diverged taxa, and taxa in the process of divergence and speciation (Avise, 2000; Funk & Omland, 2003), has already been highlighted in studies focusing on the genus *Merodon* (Mengual *et al.*, 2006; Milankov *et al.*, 2008). Distinguishing among intraspecific variation, interspecific introgression, and incomplete sorting is difficult in the case of *M. avidus*. Although much of the genetic variability could be natural variability occurring within morphological species, the observed mtDNA haplotype diversity might indicate the existence of intraspecific genetic groups, and/or of broader geographic subdivision [e.g. incomplete lineage sorting of ancestrally polymorphic allelic populations, Funk & Omland (2003); or hybridization during co-occurrence of cryptic taxa on the Dubašnica and Pindos Mts]. Sharing common alleles suggests recent divergence of taxa with ongoing gene flow or recent ancestry.

Relatively high intraspecific divergence and phenotypic substructuring detected within *M. avidus* A indicated that these diverged populations might be currently undescribed sibling species (similar levels of divergence have been reported in other studies of closely related species in the genus *Merodon*,

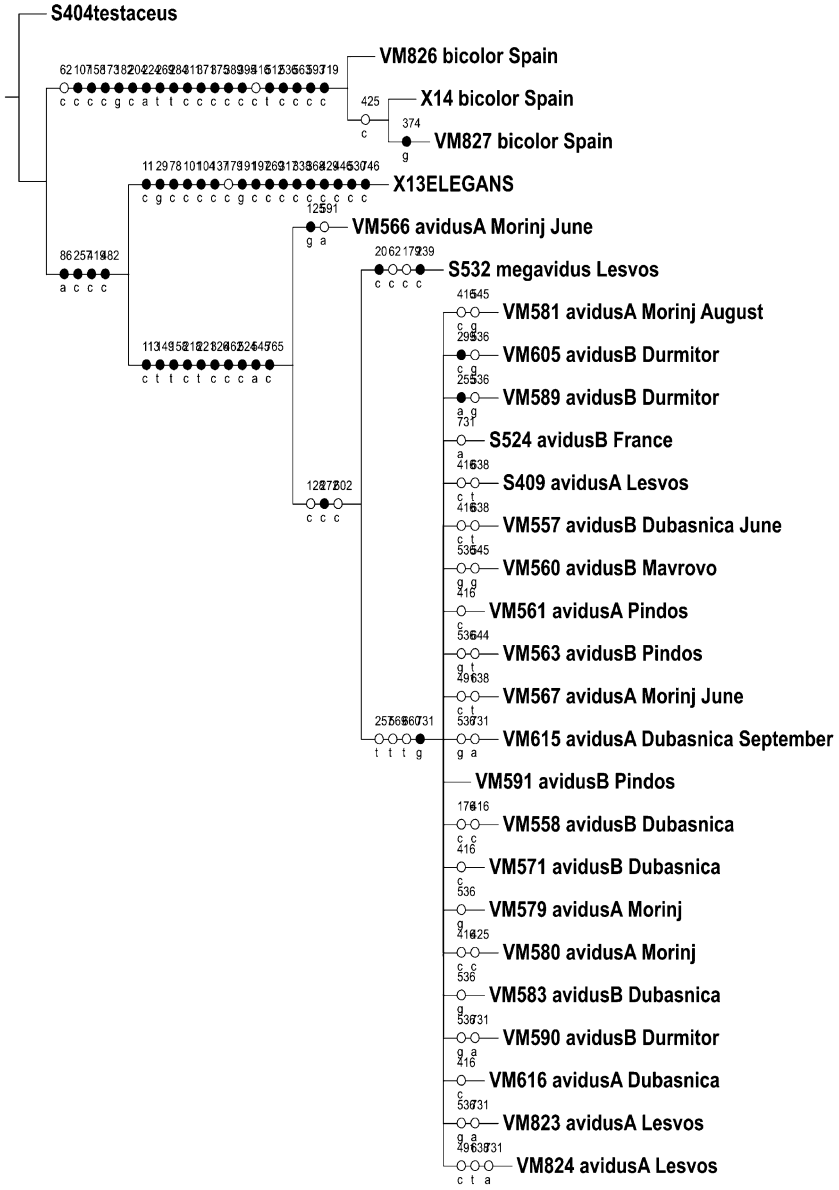

Figure 8. Strict consensus of > 200 equally parsimonious trees, length 116 steps, consistency index = 0.81, retention index = 0.87. Filled circles, nonhomoplasious changes; open circles, homoplasious changes.

Milankov *et al.*, 2008). Previously reported subdivision among geographically separated populations, local inbreeding, and deficit of heterozygotes indicated that population structuring of *M. avidus* A was a result of genetic drift and limited gene flow among fragmented populations (Milankov, Ludoški & Vujić, 2004a, b). Moreover, we found evidence of temporal divergence within Mediterranean populations of *M. avidus* A (Lesvos, Greece and Morinj, Montenegro). As specimens collected in April in Lesvos and Morinj shared haplotypes with *M. avidus* B populations from Dubašnica and Durmitor Mts, we hypothesize that the early spring generation of *M. avidus* is *M. avidus* B, whereas the summer and autumn generation represents *M. avidus* A. There are also two specimens of uncertain status, one from Lesvos (haplotype XVII) and another from Morinj (haplotype XII), that are genetically clearly distinct from each other ($P = 1.4\%$) and from members of the *M. avidus* A and *M. avidus* B lineages (Table 4; Fig. 7), although no morphological diagnostic traits support their distinctness. Finally, based on four unique and three rare alleles at allozyme loci (Milankov *et al.*, 2001), distinct wing shape, and six unique mtDNA haplotypes, we hypothesize that the spatially isolated population of *M. avidus* A from Morinj might be an evolutionarily

Table 4. Number of fixed differences (lower matrix) and uncorrected (*P*; %) distance matrix for haplotypes of *Merodon bicolor* (XVIII, XIX, XX) and the *Merodon avidus* taxa (*M. avidus* A, *M. avidus* B in bold, common haplotypes underlined) (*sequences obtained by J+I primers; Lunt *et al.*, 1996)

	I	II	III*	IV	IV/V*	**V**	VI	VII	VIII	**IX**	**X**	**XI**	XII	**XIII**	XIV	**XV**	XVI	XVII	XVIII	XIX	XX
I		0.52	0.59	0.52	0.20	0.52	0.13	0.39	0.26	0.52	0.52	0.52	1.43	0.52	0.39	0.39	0.52	1.56	5.73	5.58	5.84
II	4		0.20	0.52	0.20	0.26	0.65	0.13	0.26	0.52	0.52	0.52	1.44	0.26	0.39	0.39	0.26	1.56	5.35	5.21	5.47
III*	3	1		0.39	0.38	0.38	0.58	0.20	0.20	0.58	0.58	0.38	1.13	0.39	0.38	0.39	0.39	1.58	5.84	5.63	6.00
IV	4	4	2			0.26	0.39	0.39	0.52	0.26	0.26	0.26	1.70	0.52	0.13	0.13	0.52	1.30	5.34	5.19	5.45
IV/V*	1	1	2				0.20	0.20	0.20	0.20	0.20		0.75	0.39			0.39	1.19	5.84	5.63	6.00
V	4	4	2	2			0.65	0.39	0.52	0.26	0.26	0.26	1.43	0.52	0.13	0.39	0.52	1.56	5.47	5.32	5.58
VI	1	5	3	3	1	5		0.52	0.39	0.64	0.65	0.65	1.30	0.65	0.53	0.26	0.65	1.43	5.60	5.45	5.71
VII	3	1	1	3	1	3	4		0.13	0.39	0.39	0.39	1.30	0.13	0.26	0.26	0.13	1.43	5.34	5.19	5.45
VIII	2	2	1	4	1	4	3	1		0.54	0.54	0.54	1.48	0.26	0.39	0.41	0.26	1.62	5.52	5.32	5.58
IX	4	4	3	2	1	2	5	3	4		0.26	0.26	1.43	0.52	0.13	0.39	0.52	1.56	5.60	5.45	5.71
X	4	4	2	2	1	2	5	3	4	2		0.26	1.44	0.52	0.13	0.40	0.52	1.56	5.61	5.47	5.73
XI	4	4	2	2	0	2	5	3	4	2	2		1.43	0.52	0.13	0.39	0.52	1.56	5.60	5.45	5.71
XII	11	11	6	9	4	11	10	10	11	11	11	11		1.43	1.31	1.04	1.43	1.43	5.08	4.93	5.19
XIII	4	2	2	4	2	4	5	1	3	4	4	4	10		0.39	0.39	0.26	1.56	5.47	5.32	5.58
XIV	3	3	2	1	0	1	4	2	1	1	1	1	10	3		0.26	0.39	1.44	5.37	5.32	5.49
XV	3	3	2	1	0	3	2	2	3	3	3	3	8	12	2		0.39	1.17	5.34	5.19	5.45
XVI	4	2	2	4	2	4	5	1	2	4	4	4	11	2	3	3		1.56	5.21	5.32	5.32
XVII	11	12	8	10	6	12	11	11	12	12	12	12	11	12	11	9	12		5.21	5.06	5.32
XVIII	44	41	30	41	30	42	43	41	41	43	43	43	39	42	40	41	40	31		0.13	0.13
XIX	43	40	29	40	29	41	42	40	40	42	42	42	38	41	41	40	41	39	1		0.26
XX	45	42	31	42	31	43	44	42	42	44	44	44	40	42	43	42	41	40	1	2	

independent entity within the *M. avidus* group. Accurate description and delimitation of species boundaries of samples from Morinj and questionable specimens will be the objects of further research.

This is the first study that examines the usefulness of wing landmarks for species delimitation and for quantification of intra- and interspecific variation in hoverfly species. Based on wing size, *M. bicolor, M. avidus* A, and *M. avidus* B were clearly delimited. Data suggested that *M. avidus* groups from the Iberian and Balkan Peninsulas radiated in two distinct species groups independently, which is not surprising given the geographical distance. Herein, *M. avidus* B from Spain is reclassified as *M. bicolor* and identified as an evolutionarily independent lineage within the *M. avidus* group based on distinct COI mtDNA haplotypes, and wing shape and size. In spite of the high morphological similarity of *M. bicolor* and *M. avidus*, there is a great genetic distinction between the two taxa. *Merodon bicolor* and *M. avidus* lineages possess distinct COI haplotype patterns and each of these represents mitochondrial diversity. Both selection in different environments and historical biogeographical processes may have been important in modelling the population structure, genetic, and phenotypic structuring of the two lineages. However, only a limited number of specimens was available from Spain, and none from regions between the Balkan and Iberian Peninsulas, where a wide range of variability is to be expected. Hence, more work is required before questions about the taxonomic and phylogenetic diversity of this insect group can be answered.

In summary, the case of the *M. avidus* complex highlights the importance of the integration of multiple characters (mitochondrial sequence data and quantitative traits) in the delineation and identification of significant units of biodiversity. Previously known as a widespread species, *M. avidus* actually comprises several cryptic species, some with very restricted distributions. Two allopatric lineages, Spanish *M. bicolor* and Balkan *M. avidus,* split early, followed by diversification in each lineage. Within the *M. avidus* lineage from the Balkan Peninsula, allopatric populations of the allozyme-defined species *M. avidus* A (all populations from FYR MACEDONIA except the one from Mavrovo, populations from Pannonian region), *M. avidus* B (from Durmitor, Stara, and Kopaonik Mts), and the genetically unique taxa (from Mavrovo and Morinj), suggested vicarious differentiation from a widespread common ancestor. These results highlight the Balkan Peninsula as an area of genetic diversity, species richness, and endemism, as well as an area of evolutionary origin and a centre of biodiversity that should be addressed through conservation management.

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