Conserv Genet (2008) 9:1125–1137
DOI 10.1007/s10592-007-9426-8

RESEARCH ARTICLE

Genetic diversity of populations of *Merodon aureus* and *M. cinereus* species complexes (Diptera, Syrphidae): integrative taxonomy and implications for conservation priorities on the Balkan Peninsula

Vesna Milankov · Gunilla Ståhls · Jelena Stamenković · Ante Vujić

Received: 25 March 2007 / Accepted: 6 September 2007 / Published online: 29 September 2007
© Springer Science+Business Media B.V. 2007

Abstract The genetic structure of 10 populations of the *Merodon aureus* group from the Balkan Peninsula was examined through allozyme electrophoresis and mitochondrial DNA sequencing of the cytochrome *c* oxidase subunit I (COI). Six diagnosable cryptic taxa were identified within the morphologically defined species *M. aureus* Fabricius, 1805 and *M. cinereus* (Fabricius, 1794), with clear separation of the populations (((*M. aureus* A + *M. aureus* B) + *cinereus* complex) + *M. aureus* C). The parsimony analysis of COI sequence data of the *aureus–cinereus* complex using *Merodon avidus* A species as an outgroup resulted in two main clades, (*M. aureus* A + *M. aureus* B) and ((*M. aureus* C + *M. cinereus* B + *M. cinereus* C) + *M. cinereus* A), which differed on average by 5.7%. The observed spatial distribution of the taxonomic diversity of the group suggested that these taxa originated from a common ancestral population in the Mediterranean. Identification of genetic uniqueness and genetic endemism emphasizes the importance of molecular markers and estimation of genetic diversity in recognition of conservation units. The primary goals of the conservation measures that we propose are the protection of phylogenetic lineages within the highly diverse *M. aureus* group taxa and conservation of the genetic variation through management of important areas.

V. Milankov (✉) · J. Stamenković · A. Vujić
Department of Biology and Ecology, University of Novi Sad, Trg Dositeja Obradovića 2, Novi Sad 21000, Serbia
e-mail: vesnam@ib.ns.ac.yu

G. Ståhls
Finnish Museum of Natural History, University of Helsinki, PO Box 17, Helsinki 00014, Finland

Keywords Allozymes · MtDNA · Cryptic taxa · Genetic units · Genetic diversity · Conservation · Diptera · *Merodon*

Introduction

The genus *Merodon* is the second largest genus of Palaearctic hoverflies (Diptera, Syrphidae) with more than 50 European species (Speight 2004), and the highest diversity in the Mediterranean region. The high endemicity on the Balkan (Vujić et al. unpublished) and Iberian (Marcos Garcia et al. 2007) Peninsulas is probably due to isolation of different districts during the Pleistocene glaciations. Studying phylogenetic relationships within, and genetic distinctiveness of the genus *Merodon* is challenging due to the high diversity, presence of cryptic species, taxonomic uncertainties, and unclear relationships among taxa.

An example of the great morphological variability exhibited in the genus *Merodon* is the *aureus* group of taxa with bi-colored tibiae, mentioned under two names in recent literature (Šimić and Vujić 1996; Van de Weyer and Dils 2002) for the central and south-eastern Europe: *Merodon aeneus* Megerle in Meigen, 1822 and *M. cinereus* (Fabricius, 1794). In the Biosystematic Database of World Diptera (BDWD), Thompson (2005) cited *Merodon aureus* as a senior synonym of *M. aeneus*, and we accept this synonymy. There are six more synonyms of *M. aureus* and four synonyms for *M. cinereus* cited in Peck (1988). Morphologically similar to *M. aureus* is a European taxon *Merodon funestus* (Fabricius, 1794). Populations of *M. funestus* also exhibit morphological variation in the length of antennae and leg coloration.

For identification of areas of genetic endemism of taxonomically diverse and challenging taxa, such as the

genus *Merodon*, it is important to delineate genetic units that are geographically discrete, often characterized by non-overlapping haplotype distributions and/or occurrence of unique allozyme markers (Moritz 1994). For example, the presence of unique alleles is a marker of distinct genetic units, implying existence of cryptic taxa with reproductive incompatibility, while species-specific alleles and their combinations in spatially subdivided populations probably reflect their origin in past events of gene pool fragmentation. However, different allozyme loci can diverge at different rates and the rate of divergence can vary through time, making it difficult to allocate a time since divergence based on genetic distance (Thorpe 1982). Molecular data of both nuclear and mitochondrial genes are a valuable source of information in taxonomic and evolutionary studies in hoverflies, especially when morphological characters are insufficient or not exclusive enough for identification of morphologically cryptic taxa (e.g. Milankov et al. 2005; Rojo et al. 2006).

The first goal of this study was to identify diagnosable taxa within available populations of the *aureus* group occurring on the Balkan Peninsula using allozyme markers and mitochondrial DNA cytochrome *c* oxidase subunit I gene (mtDNA COI) sequences. The second aim was to quantify genetic variability in the populations of *M. aureus* and *M. cinereus* complexes and in *M. funestus* as a measure of an evolutionary potential, and analyze genetic and phylogenetic relationships among the taxa. Finally, as there are no plans that focus on the management of threatened hoverflies species on the Balkan Peninsula, conservation implications for the preservation of the genetic diversity of these taxa based on our findings are discussed.

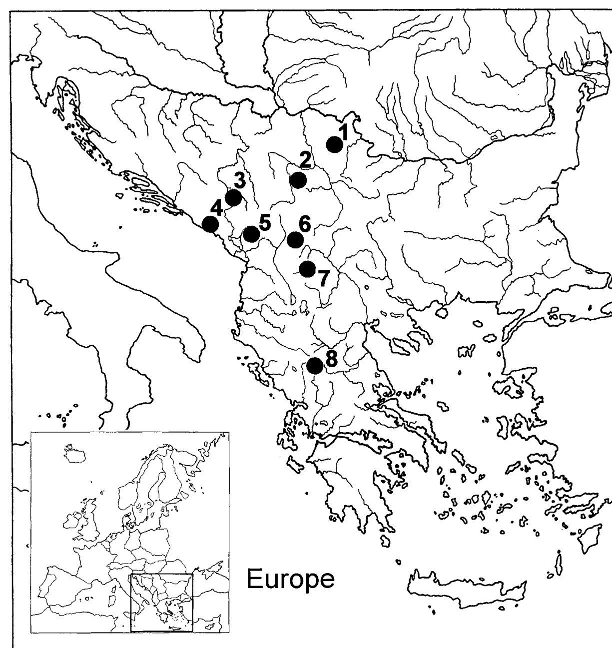

Fig. 1 Map of the Balkan Peninsula. Origin of the analyzed populations: 1. Dubašnica Mountain (DUB Mt), E 21°59′, N 44°01′ (Serbia); 2. Kopaonik Mountain (KOP Mt), E 20°40′, N 43°15′ (Serbia); 3. Durmitor Mountain (DUR Mt), E 19°00′, N 43°11′ (Montenegro); 4. Morinj (MOR), E 18°40′, N 43°29′30″ (Montenegro); 5. Prokletije Mountain (PRO Mt), E 19°50′, N 42°32′ (Serbia); 6. Šar planina Mountain (ŠAR Mt), E 21°05′, N 42°12′ (Serbia). 7. Mavrovo Lake (MAV Lk), E 20°44′30″, N 41°38′30″ (FYR Macedonia); 8. Pindos Mountain (PIN Mt), E 20°37′, N 39°14′ (Greece). Locality Morinj is the territory with intermixed evergreen Mediterranean maritime woodlands and maquis and Submediterranean oak woodlands at the Adriatic Sea in the Mediterranean area. Kopaonik, Durmitor, Prokletije and Šar-planina are high Dinaric mountains with different types of biomes, from deciduous woodlands at low altitudes (up to 700 m), European coniferous boreal woodlands at the higher altitudes, to the biome of alpine and high Nordic rock-grounds pastures and snow patches in the highest zone

Material and methods

Sample collection

Samples of 10 populations of the *Merodon aureus* group and *M. funestus* were collected from five regions (Fig. 1) on the Balkan Peninsula over multiple years (population code and number of collected specimens are given in Table 1). In addition, populations of *M. avidus*, comprising cryptic taxa *M. avidus* A and *M. avidus* B, from five different biogeographic regions on the Balkan Peninsula were sampled to be used as an outgroup (Milankov et al. 2001) (Table 1).

Specimens were initially identified to either the *aureus* or *cinereus* complex based on the morphology. Specimens from the *M. aureus* and *M. cinereus* complexes have dark legs except for pale knees, both ends of tibiae and tarsi in part. Pale hairs on thorax and abdomen, dull scutum and rough punctuation of scutum and tergites are characteristic for the *M. aureus* complex, contrary to dark hairs on thorax and abdomen, shiny scutum and finer punctuation of scutum and tergites in the *M. cinereus* complex.

Allozyme analysis

All sampled populations (a total of 193 specimens) were included in the allozyme analysis in order to determine molecular markers that could be used to identify the studied taxa (Table 1). Allozyme data of the outgroup, *M. avidus* (Milankov et al. 2001) were used for constructing a rooted dendrogram.

Genetic variability was studied by standard 5% polyacrylamide gel electrophoresis, following Munstermann (1979) (FUM, GPD, GPI, HAD, HK, IDH, MDH, ME, PGM, SOD) and Pasteur et al. (1988) (AAT), with slight

Table 1 Species, population code and collection location of samples included in this study

Species	Locality	Locality code	*n*	Sample code	GenBank accession No.	Collection date	Collectors
M. aureus A	Durmitor Mt.	DUR	11	VM351-COI	DQ387912	20/06/1998	Milankov, V., Tanurdžić, M., Radović, D., Vujić, A.
				VM407-COI	DQ387919		
	Morinj	MOR	27	VM326-COI	DQ387906	16/05/1998	Dragišić, S., Mićić R., Radenković, S., Vujić, A.
				VM354-COI	DQ387913		
M. aureus B	Kopaonik Mt.	KOP	23	VM333-COI	DQ387908	06/06/1998	Vujić, A.
				VM348-COI	DQ387910		
				VM347-COI	DQ387921	20/06/1998	Milankov, V., Tanurdžić, M., Radović, D., Vujić, A.
				VM332-COI	DQ387907		
M. aureus C	Durmitor Mt.	DUR	21	VM349-COI	DQ387911	28/07/1998	Milenković, D., Vujić, A.
				VM366-COI	DQ387917		
	Morinj	MOR	6	VM340-COI	DQ387909	21/08/1997	Milankov, V., Vujić, A.
				VM355-COI	DQ387914		
				VM428-COI	DQ387920	25/09/1997	Vujić, A.
M. cinereus A	Kopaonik Mt.	KOP	37	VM313-COI	DQ387902	15/08/1997	Milankov, V., Milenković, D., Stamenković, J., Vujić, A.
				VM314-COI	DQ387903		
M. cinereus B	Durmitor Mt.	DUR	37	VM315-COI	DQ387904	28/07/1998	Milenković, D., Vujić, A.
				VM316-COI	DQ387905		
	Prokletije Mt.	PRO	4	VM406-COI	DQ387918	26/07/1994	Milankov, V., Radenković, S., Radišić, P., Radnović, D., Vujić, A.
M. cinereus C	Šar planina Mt.	ŠAR	5	VM362-COI	DQ387915	16/07/1995	Radenković, S., Radišić, P., Vujić,A.
				VM363-COI	DQ387916		
M. funestus	Morinj	MOR	22	VM338-COI	DQ387901	31/08/1997	Milankov, V., Vujić, A.
				VM458-COI	DQ387900	16/05/1998	Dragišic, S., Mićić R., Radenković, S., Vujić, A.
M. avidus A[a]	Morinj	MOR	30	VM566-COI	DQ387897	18/06/1998	Milenković, D., Vujić, A.
				VM581-COI	DQ387896	31/08/1998	Milankov, V., Vujić, A.
	Dubašnica Mt.	DUB	14	–		06/97; 08/97; 09/97	Dević, D., Milankov V., Milenković, D., Radenković,S., Šimić,S.,Vujić,A.
	Pindos Mt.	PIN	9	–		20/05/1997	Radenković, S., Šimić, S.,Vujić, A.
M. avidus B[a]	Dubašnica Mt.	DUB	21	–		06/97; 08/97; 09/97; 07/98	Dević, D., Milankov V., Milenković, D., Radenković,S., Šimić,S.,Vujić,A.
	Durmitor Mt.	DUR	9	VM589-COI	DQ387898	20/06/199820/06/1998	Milankov, V., Tanurdžić, M., Radović, D., Vujić, A.
				VM605-COI	DQ387895		
	Mavrovo Lake	MAV	17	–		10/07/1998	Radenković, S., Šimić, S.,Vujić, A.
	Pindos Mnt.	PIN	17	–		20/05/1997	Radenković, S., Šimić, S.,Vujić, A.

Number of specimens (*n*) used for allozyme analysis and sequences amplified for molecular analysis are indicated

[a] *Merodon avidus* allozyme data were published in: Milankov et al. (2001)

modifications (Milankov 2001) (Table 2). The electrophoresis of individuals from different populations was conducted in the same gel for direct interspecific comparison. Loci were numbered and alleles marked alphabetically with respect to order of increasing anodal migration. Two loci *Sod*-2 and *Sod*-3 were not available for comparison of populations of the *aureus* group and the outgroup.

Genotype and allele frequencies were calculated directly from the observed banding patterns based on the genetic interpretation of zymograms. Calculated parameters of

Table 2 Enzymes and loci investigated (EC number and name), buffer systems and tissue extract used in electrophoresis

Enzyme	Abb.	E.C. Number[a]	Loci	Buffer[b]	Tissue extract[c]
aspartat amino transferases	AAT	2.6.1.1	*Aat*	TC	head
fumarate hydratase	FUM	4.2.1.2.	*Fum*	TC	head
glycerol 3-phosphate dehydrogenase	GPD	1.1.1.8.	*Gpd*-2	TC	thorax
glucosephosphate isomerase	GPI	5.3.1.9.	*Gpi*	TBE	thorax
b-hydroxy acid dehydrogenase	HAD	1.1.1.30.	*Had*	TC	thorax
hexokinase	HK	2.7.1.1.	*Hk*-2	TBE	head
			Hk-3		thorax
isocitrate dehydrogenase	IDH	1.1.1.42.	*Idh*-2	TC	thorax
malate dehydrogenase	MDH	1.1.1.37.	*Mdh*-1	TC	head
			Mdh-2		head
malic enzyme	ME	1.1.1.40.	*Me*	TBE	head
phosphoglucomutase	PGM	2.7.5.1.	*Pgm*	TBE	thorax
superoxide dismutase	SOD	1.15.1.1.	*Sod-1*	TBE	thorax
			Sod-2		
			Sod-3		

[a] EC number—Enzyme Commision

[b] TC buffer—1 M Tris–citric buffer pH = 7.1; TBE buffer—1 M Tris–boric–EDTA pH = 8.9

[c] Added in loading buffer (20% sucrose, Triton X-100 [0.5%], Tris–citrate pH 7.0 electrode buffer and trace amount of bromphenol blue tracking dye)

population genetic structure were corrected using Levene's (1949) formula for small samples. Divergence among identified genetic units within *M. aureus* and *M. cinereus* complexes was examined using pairwise Wright's F_{ST} values (Weir 1996) as an estimate of the proportion of the total genetic variance. Statistical analyses of allozyme data were performed using the computer program BIOSYS-2 (Swofford and Selander 1989). Diagnostic value of allozymes was calculated after Ayala and Powell (1972). A locus was considered diagnostic if the probability for correct identification of individuals belonging to the *M. aureus* group was 100% (complete differentiation) or higher than 95% (frequency of a common allele in one of two species is less than 0.05). Loci with overlapping allelic frequencies of 0.15 and 0.20 (correct diagnosis with at least 85% and 80% probability, respectively), or a combination of discriminatory loci, were also used for distinguishing species.

In the pilot study of gene-enzyme varibaility, diagnostic loci and cryptic species were detected within the morphologically defined *M. aureus* and *M. cinereus* species. Based on fixed allozyme diferences associated with morphological traits (see Results), we divided sympatric populations of *M. aureus* into two putative taxa: *M. aureus* A and *M. aureus* C. Within morphologically defined *M. aureus* species, a population from Kopaonik Mt. was identified as a specific genetic unit, *M. aureus* B. Diagnostic allozyme loci allowed us to determine allopatric populations of *M. cinereus* group as the cryptic species *M. cinereus* A, *M. cinereus* B and *M. cinereus* C.

DNA sequencing

We obtained a 720 bp fragment of the mtDNA COI gene, corresponding to nucleotide positions 2233–2952 in *Drosophila yakuba* sequence (Clary and Wolstenholme 1985). A total of 24 specimens from all 10 populations of the *M. aureus* group, *M. funestus* and four specimens of the *M. avidus* outgroup from two localities (Table 1) were used for sequencing (all sequences have been deposited in GenBank, Accession numbers of the analyzed specimens are listed in Table 1). DNA was extracted from legs or other parts of the fly remaining after allozyme electrophoresis using the Nucleospin Tissue DNA extraction kit (Machery-Nagel, Düren, Germany) following the manufacturer's protocols and then re-suspended in 50 µl of ultra-pure water. Remains of specimens, including male genitalia, used for the morphological studies and for DNA extraction are deposited at the Finnish Museum of Natural History (Helsinki, Finland).

PCR reactions were carried out in 25 µl reaction aliquots containing 2 µl DNA extract, 1 µl of each primer (at 10 pmol/µl), 0.25 µl of DNA polymerase (5 U/µl), 2 µl 2.5 mM $MgCl_2$, 2.5 µl 10 × Buffer II (MBI Fermentas, St. Leon-Rot, Germany) and 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 sequencing the COI fragment (720 bp) were the forward primer C1-J-2183 (5′-CAA CAT TTA TTT TGA TTT TTT GG-3′) (alias JERRY) and the reverse primer TL2-N-3014 (5′-TCC AAT GCA CTA ATC TGC CAT ATT A-3′) (alias PAT) (Simon et al. 1994). 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, Foster City, CA, USA) at one-fourth of the recommended volumes on an ABI PRISM 377 (Applied Biosystems, Foster City, CA, USA) semi-automated DNA sequencer. The sequences were edited for base-calling errors and assembled using Sequence Navigator™ (version 1.01) (Applied Biosystems, Foster City, CA, USA).

Genetic and phylogenetic analyses

Nei's genetic distance and identity (1978) were used to compare gene frequencies among species or populations, and to construct a dendrogram using clustering with the Unweighted Pair Group Method with Arithmetic Average (UPGMA) (Sneath and Sokal 1973). Prevosti's distance (Wright 1978) was employed to estimate phylogenetic relationships through the Wagner procedure (Farris 1972) rooted tree.

Alignment of COI sequences was trivial due to lack of indels and was done by eye. Parsimony analysis was performed using NONA (Goloboff 1999) and spawn with the aid of Winclada (Nixon 2002), using heuristic search algorithm with 1000 random addition replicates (mult*1000), holding 100 trees per round (hold/100), maxtrees set to 100,000 and applying TBR branch swapping. All base positions were treated as equally weighted characters.

Results

Species boundaries

Morphological diagnostic traits

Extensive variability observed in the coloration of hairs on eyes, legs, thorax, and abdomen; and the structure of tegument on scutum and tergites, indicated possible occurrence of cryptic taxa within the analyzed species. Based on morphological characters of the studied populations of *Merodon aureus* and *M. cinereus* occurring on the Balkan Peninsula, four morphotypes could be distinguished (Table 1):

1. *Merodon aureus* A and B, morphologically inseparable: abdomen and mesonotum with dense, yellow-reddish hairs in male; female abdomen with shorter, mixed pale and black hairs, tergites II–IV with more or less clear stripes of polinosity with pale pilosity; tegument of mesonotum and tergites with golden tomentum; hind femora pale haired; at least upper half of eyes black haired. Analyzed populations were from Durmitor Mt., Morinj (*M. aureus* A DUR, *M. aureus* A MOR) and Kopaonik Mt. (*M. aureus* B KOP).
2. *Merodon aureus* C: abdomen and mesonotum with dense, yellow-reddish hairs in males; female abdomen with shorter, mixed pale and black hairs, tergites II–IV with more or less clear pollinose stripes; tegument of mesonotum and tergites with golden tomentum, but less than in *Merodon aureus* A and B: hind femora with black hairs on apical third; eyes pale haired, exceptionally with a few black hairs on dorsal corner. Analyzed populations were from Durmitor Mt. (DUR) and Morinj (MOR).
3. *Merodon cinereus* A and C, morphologically inseparable: mesonotum with pale hairs in anterior half and black in posterior (from only few to almost completely black hairs); female abdomen with shorter, mixed pale and black hairs, tergites II–IV with more or less clear pollinose stripes; tegument of mesonotum and tergites finely punctured, shiny; tergites 2 and 3 in males pale haired; tergite 4 with at least few black hairs (in *M cinereus* A usually completely black haired); female abdomen with shorter, mixed pale and black hairs, tergites II–IV with more or less clear pollinose stripes. Analyzed populations were from Kopaonik (*M cinereus* A KOP) and Šar-Planina Mts. (*M. cinereus* C ŠAR).
4. *Merodon cinereus* B: mesonotum with pale hairs in anterior half and black in posterior (from only few to almost completely black *Merodon cinereus* A and C; tegument of mesonotum and tergites finely punctured, shiny; tergites 2–4 in males pale haired with two stripes of black hairs; female abdomen with shorter, mixed pale and black hairs, tergites II–IV with more or less clear pollinose stripes. Analyzed populations were from Durmitor (DUR) and Prokletije Mts (PRO).

Taxa recognised as new to science in present study will be described in a separate taxonomic paper (Vujić et al. unpublished).

Diagnostic allozyme loci

Based on diagnostic allozyme loci and fixed allelic differences, six well-defined genetic units were recognized within the *aureus* and *cinereus* complexes. Three taxa were

delineated within the morphologically defined *M. aureus* species. Based on the alleles at the diagnostic loci (*Aat, Had, Me* and *Sod*-1) *M. aureus* C was distinguishable from *M. aureus* A and *M. aureus* B. The allele Had^m within the *M. aureus* complex defined a group comprising *M. aureus* A and *M. aureus* B. The cladogram resolved these taxa as sister groups. A specific allele of diagnostic significance (Pgm^c) was observed in the population of the cryptic species *M. aureus* B from Kopaonik Mt. (Table 3). Diagnostic allozyme loci allowed identification of allopatric populations of *M. cinereus* group as cryptic species *M. cinereus* A, *M. cinereus* B and *M. cinereus* C. Identification and delimitation of *M. cinereus* A and *M. cinereus* B was possible with a 100% probability based on the genotypes at the *Had* locus. Furthermore, populations could be identified based on the specific allelomorphs at the *Gpi* and *Me* loci. Alleles Gpi^k and Me^l defined *M. cinereus* B PRO and *M. cinereus* C ŠAR populations, respectively (Table 3).

Unique alleles were also used to assess the spatial variability between conspecific populations and interpopulation variation. Some private alleles registered within the *M. aureus* complex (Me^j, Me^m, Pgm^c, Fum^c, Gpi^i, Had^n) were common alleles with *M. desuturinus* Vujić, Šimić et Radenković, 1995 (Milankov et al. unpublished), *M. cinereus* B and/or *M. funestus*. Likewise, some unique alleles within the *M. cinereus* complex (Aat^a, Fum^c, Gpi^a, Had^n, *Mdh*-2^g, Me^i, Pgm^h and *Sod*-1^e) were shared with *M. aureus* A, *M. aureus* B, *M. funestus, M. aureus* C and/or *M. desuturinus* (Milankov et al. unpublished).

Mitochondrial COI diagnostic haplotypes

A total of 10 haplotypes were found in 22 analyzed individuals. There were 86 variable positions defining haplotypes. Haplotypes formed two groups of the *aureus* and *cinereus* complexes that differed from one another by 38 or more nucleotide changes. One haplotype corresponded to *M. aureus* B (haplotype III), one to *M. aureus* C MOR, *M. cinereus* B and *M. cinereus* C (haplotype VII), one to *M. cinereus* A (haplotype VIII), two to *M. aureus* A (I and II haplotypes), two to *M. funestus* (IX and X haplotypes) and four to *M. aureus* C (IV, V, VI and VII haplotypes), which were subdivided into two main lineages (Fig. 2).

Genetic diversity and divergence

Population genetic structure

A total of 44 allozymes were registered in all populations and across all loci. Within the *aureus* complex of populations, the greatest number of alleles was observed at Morinj locality (Table 3). Among the populations from the *cinereus* complex, *M. cinereus* A KOP had the greatest number of allelomorphs (Table 3). The largest number of alleles and genotypes were identified at the *Me* (6) and *Had* (7) loci, respectively (Table 3).

Analysis of parameters of population genetic structure showed differences among the analyzed species (Table 4). The mean number of alleles per locus (*A*) ranged from 1.1 to 1.5, and frequency of polymorphic loci (*P*) was the lowest in the population of *M. cinereus* C and the highest in *M. aureus* C and *M. funestus*. Out of 15 analyzed loci, only *Mdh*-1. *Sod*-2 and *Sod*-3 isozyme loci were monomorphic (with a common allele) in all 10 studied populations. Average observed heterozygosity (H_o) in all populations was lower than expected heterozygosity (H_e) (Table 4). Heterozygous genotypes were identified only at the *Gpi, Had* and *Sod*-1 loci, however none were registered in populations of *M. aureus* B and *M. funestus*. At the *Gpi* locus genotype $Gpi^{a/j}$ (0.045 frequency) was noted in *M. cinereus* B DUR. A common heterozygote $Had^{m/q}$ was detected in *M. cinereus* A KOP (0.459) and *M. cinereus* C ŠAR (0.200) populations as well as one unique $Had^{n/r}$ in *M. cinereus* B DUR (0.027). *M. aureus* A DUR and *M. aureus* A MOR, and *M. aureus* C DUR and *M. aureus* C MOR had species-specific heterozygotes at *Sod*-1 locus (*Sod*-$1^{f/h}$: 0.429 and 0.400, *Sod*-$1^{d/e}$: 0.313 and 0.500, respectively). Temporal distribution of alleles at the *Gpi*, *Me* (allele Gp^k, Me^k, Me^i in "summer" generation), *Had, Idh*-2 and *Sod*-1 loci (Had^q, *Idh*-2^b, *Sod*-1^e in "spring" generation) was observed in the *M. funestus* MOR population.

Interspecific divergence

The greatest average genetic distance (0.89 ± 0.07) was between *M. funestus* and the group consisting of taxa of the *aureus* and *cinereus* complexes (group II in Fig. 3). The average genetic distance among the analyzed *aureus* and *cinereus* taxa was 0.22 ± 0.07 (group I). Within the *aureus* complex, two lineages were observed: closely related *M. aureus* A and *M. aureus* B species (average genetic distance within this lineage was 0.06 ± 0.05), and *M. aureus* C (0.33 ± 0.02 distance from the *M. aureus* A and *M. aureus* B lineage). When the outgroup was included, the highest *D*-value was detected for *avidus* populations and the *M. funestus* species comparison (cluster IV: 1.99 ± 0.08) (Fig. 3). Analysis of allozyme variability and genetic relationships among taxa of the *aureus* group and *avidus* outgroup found common alleles and genotypes at the *Gpi, Hk*-2, *Hk*-3, *Mdh*-2 and *Pgm* loci, and the *M. aureus* A species was the closest to subgeneric outgroup populations (D = 0.86–1.01). Indeed, the UPGMA

Table 3 Total number of detected alleles (*n* = number of analyzed individuals) and allelic frequency at variable loci in the *M. aureus* group and *M. funestus* (common alleles with *M. avidus* outgroup are underlined)

Species		*M. aureus* A		*M. aureus* B	*M. aureus* C		*M. cinereus* A	*M. cinereus* C	*M. cinereus* B		*M. funestus*
Population[c]		DUR	MOR	KOP	DUR	MOR	KOP	ŠAR	DUR	PRO	MOR
Locus	Allele										
Aat	A[b]	–	–	–	–	–	–	–	0.027[a]	–	–
	B	1.000	1.000	1.000	0.200	0.167	1.000	1.000	0.973	1.000	–
	C	–	–	–	0.800	0.833	–	–	–	–	1.000
Fum	A	0.909	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.750	–
	C	0.091[a]	–	–	–	–	–	–	–	0.250[a]	1.000
	E	–	–	–	–	–	–	–	–	–	–
Gpd-2	B	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	–
	D[b]	–	–	–	–	–	–	–	–	–	1.000
	H	–	–	–	–	–	–	–	–	–	–
Gpi	A[b]	–	–	–	–	–	–	–	0.023[a]	–	–
	I	–	–	–	–	0.167[a]	–	–	–	–	0.300
	J	0.727	0.684	0.526	1	0.500	0.909	0.800	0.977[a]	–	0.600
	K	0.273	0.316	0.474		0.333[a]	0.091	0.200	–	1.000[a]	0.100
Had	H	–	–	–	–	–	–	–	–	–	–
	M	1.000	1.000	1.000	–	–	0.716	0.500	–	–	–
	N	–	–	–	0.059[a]	–	–	–	0.014[a]	–	0.882
	Q	–	–	–	0.941	1.000	0.284	0.500	–	–	0.118
	R[b]	–	–	–	–	–	–	–	0.986	1.000	–
Hk-2	A[b]	–	–	–	–	–	–	–	–	–	1.000
	C	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	
Hk-3	A[b]	-	–	–	–	–	–	–	–	–	1.000
	C	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	
Idh-2	A	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.818
	B[b]	–	–	–	–	–	–	–	–	–	0.182
Mdh-2	E	1.000	0.962	1.000	1.000	0.750	1.000	1.000	1.000	0.500	
	G	–	0.038[a]	–	–	0.250[a]	–	–	–	0.500[a]	
	I[b]	–	–	–	–	–	–	–	–	–	1.000
Me	I	0.909	1.000	0.870	–	–	0.194	–	–	–	0.050
	J[b]	–	–	0.043	–	–	–	–	–	–	–
	K	0.091[a]	–	0.087	0.875	0.400	0.722	–	1.000	0.333	0.300
	L	–	–	–	0.125	0.400	0.083	1.000	–	0.667[a]	–
	M	–	–	–	–	0.200[a]	–	–	–	–	0.650
Pgm	C[b]	–	–	1.000	–	–	–	–	–	–	–
	F	1.000	1.000	–	0.267[a]	–	0.568	1.000	1.000	1.000	1.000
	H	–	–	–	0.733	1.000	0.432	–	–	–	–
Sod-1	D	–	–	–	0.281	0.250	1.000	1.000	1.000	0.667	–
	E	–	–	–	0.719	0.750	–	–	–	0.333[a]	0.053
	F	0.857	0.523	1.000	–	–	–	–	–	–	0.947
	H[b]	0.143	0.477	–	–	–	–	–	–	–	–
# Alleles		17	18	18	20	22	20	17	18	19	22
(*n*)		11	17	23	21	6	37	5	37	4	22

[a] Locality specific alleles within a species

[b] Unique alleles allowing species delineation

[c] DUR—Durmitor Mt, MOR—Morinj, KOP—Kopaonik Mt, ŠAR—Šar planina Mt, PRO—Prokletije Mt

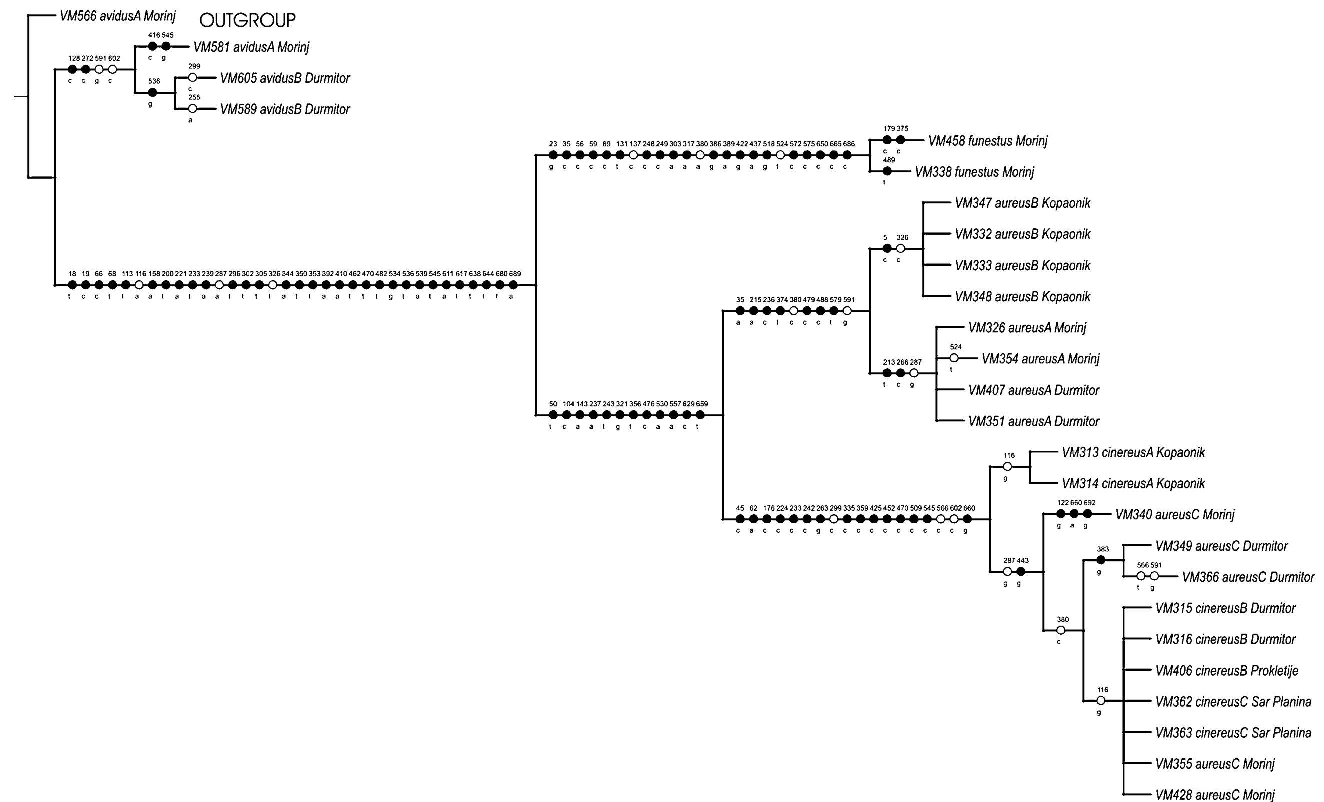

Fig. 2 The most parsimonious tree based on mitochondrial data, length 158 steps, CI = 0.87, RI = 0.96. Filled circles = nonhomoplasious changes, open circles = homoplasious changes

dendrogram using Nei's Genetic Distance (1978) and Wagner procedure dendrogram using Prevosti's distance (Figs. 4, 5) supported the closest relationship of *M. aureus* A and *M. aureus* B species pair: (((*M. aureus* A + *M. aureus* B) + *cinereus* complex) + *M. aureus* C), while *M. funestus* was the most divergent species in comparison with taxa of the *aureus* and *cinereus* complexes.

Genetic differentiation among newly defined taxa within the *M. aureus* and *M. cinereus* complexes was quantified by F_{ST} parameter as well. Genetic divergence between *M. aureus* A and *M. aureus* B ($F_{ST} = 0.48$) resulted from the presence of species-specific alleles at the *Pgm* locus ($F_{ST} = 0.48$) and variance of the allelic frequencies at *Sod*-1 ($F_{ST} = 0.36$), *Me* ($F_{ST} = 0.14$) and *Fum* ($F_{ST} = 0.05$). Greater value of genetic differentiation ($F_{ST} = 0.73$) was observed on the level of the *aureus* complex and was caused by allelic differences of *Had* ($F_{ST} = 0.95$), *Pgm* ($F_{ST} = 0.84$), *Sod*-1 ($F_{ST} = 0.76$), *Aat* ($F_{ST} = 0.76$), *Me* ($F_{ST} = 0.66$), *Gpi* ($F_{ST} = 0.31$) and *Mdh*-2 ($F_{ST} = 0.29$) loci. Among populations of the *cinereus* complex the observed F_{ST} value ($F_{ST} = 0.59$) was a result of the allelic frequency differences at *Had* ($F_{ST} = 0.74$), *Gpi* ($F_{ST} = 0.60$), *Mdh*-2 ($F_{ST} = 0.59$), *Me* ($F_{ST} = 0.51$), *Sod*-1 ($F_{ST} = 0.40$), *Pgm* ($F_{ST} = 0.38$) and *Fum* ($F_{ST} = 0.26$) loci.

MtDNA diversity and parsimony analysis

Sequence divergences (uncorrected *p* divergence in %) within and between conspecific populations ranged from 0.14% (between haplotypes I and II in *M. aureus* A MOR), 0.29% (IV and V in *M. aureus* C DUR), 0.42% (IX and X in *M. funestus* MOR) to 0.85% (VI and VII in *M. aureus* C MOR) and from 0.14% (*M. aureus* A) to 0.28–1.13% (*M. aureus* C), respectively. Within the *aureus* A + *aureus* B clade there was 1.19% divergences on average. The unique haplotype of *M. cinereus* C (VIII) differed from (*M. aureus* C + *M. cinereus* B + *M. cinereus* C) clade by 0.75% on average. Haplotype VII was the most common and widely distributed (*M. aureus* C MOR, *M. cinereus* C ŠAR, *M. cinereus* B DUR, *M. cinereus* B PRO) and differed from other members of its lineage by two (IV haplotype) to six (VI) and from another lineage by 40 (III) to 43 (II) nucleotide changes. Within the *aureus* group as a whole, haplotypes of *M. funestus* (IX, X) differed the most by 52 (II) to 71 (V) compared with the members of the *aureus* and *cinereus* complexes (Table 5). Sequence divergence between *M. funestus* and *aureus*/*cinereus* complexes as well as between *M. avidus* group and *M. aureus* group averaged 8.87% and 10.39%, respectively (Table 5). Substitutions were most abundant at the third-codon position (82.80%)

Table 4 Estimates of genetic structure of the *Merodon aureus* group and *M. funestus* (DUR—Durmitor Mt, MOR—Morinj, KOP—Kopaonik Mt, ŠAR—Šar planina Mt, PRO—Prokletije Mt)

	M. aureus A		*M. aureus* B	*M. aureus* C		*M. cinereus* A	*M. cinereus* B		*M. cinereus* C	*M. funestus*
Locus	DUR	MOR	KOP	DUR	MOR	KOP	DUR	PRO	ŠAR	MOR
Aat	–	–	–	+	+	–	x	–	–	–
Fum	+	–	–	–	–	–	–	+	–	–
Gpd-2	–	–	–	–	–	–	–	–	–	–
Gpi	+	+	+	–	+	+	x	–	+	+
Had	–	–	–	+	–	+	x	–	+	+
Idh-2	–	–	–	–	–	–	–	–	–	+
Mdh-2	–	x	–	–	+	–	–	+	–	–
Me	+	–	+	+	+	+	–	+	–	+
Pgm	–	–	–	+	–	+	–	–	–	–
Sod-1	+	+	–	+	+	–	–	+	–	+
n (SE)	9.5 (0.4)	21.7 (1.0)	20.2 (0.6)	16.2 (0.2)	4.7 (0.2)	31.2 (1.6)	30.9 (1.6)	3.7 (1.3)	4.9 (0.1)	19.5 (0.5)
Mean	15.6			10.45			17.3			
U	2	1	–	2	4	–	3	5	–	–
A (SE)	1.3 (0.1)	1.2 (0.1)	1.3 (0.2)	1.3 (0.1)	1.5 (0.2)	1.3 (0.2)	1.2 (0.1)	1.3 (0.1)	1.1 (0.1)	1.5 (0.2)
Mean	1.25			1.4			1.25			
$P_{(0.95)}$	0.267	0.133	0.200	0.333	0.333	0.267	0.000	0.267	0.133	0.333
Mean	0.200			0.333			0.134			
$P_{(0.99)}$	0.267	0.200	0.200	0.333	0.333	0.267	0.200	0.267	0.133	0.333
Mean	0.234			0.333			0.234			
H_0 (SE)	0.019 (0.019)	0.027 (0.027)	0.057 (0.036)	0.021 (0.021)	0.033 (0.033)	0.031 (0.031)	0.005 (0.003)	0.000	0.013 (0.013)	0.000
Mean	0.023			0.027			0.003			
H_e (SE)	0.065 (0.032)	0.067 (0.042)	0.057 (0.036)	0.096 (0.040)	0.152 (0.062)	0.100 (0.047)	0.008 (0.005)	0.118 (0.052)	0.055 (0.038)	0.109 (0.048)
Mean	0.066			0.124			0.063			

n = Mean sample size per locus; SE = standard error; A = Mean number of alleles per locus; P = Frequency of polymorphic loci based on the criterion of 0.95 (+); 0.99 (x); monomorphic locus (–); H_o = Average frequency of observed heterozygosity; H_e = Average frequency of expected heterozygosity

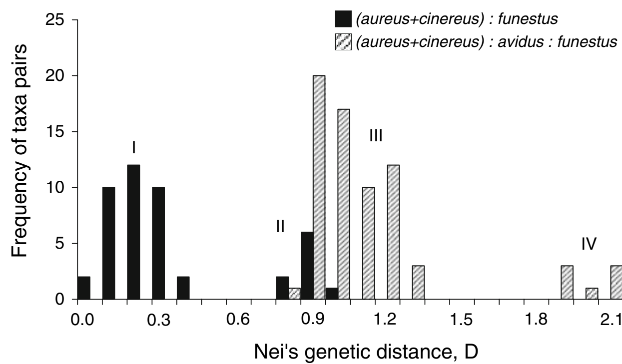

Fig. 3 Genetic distance among populations of the *M. aureus* and *M. cinereus* complexes and *M. funestus* (black bars) and when *M. avidus* out group was included (patterned bars). *M. funestus* clearly separates from the (*M. aureus* + *M. cinereus*) group (II: average $D = 0.89 \pm 0.06$) while the average D within the *M. aureus* + *M. cinereus* group is 0.21 ± 0.1 (I). The average distance within the *M. aureus* complex (0.22 ± 0.15) is larger than within the *M. cinereus* complex (0.12 ± 0.05). The average genetic distance from the outgroup *M. avidus* is the largest for *M. funestus* (IV: 1.99 ± 0.07), while the (*M. aureus* + *M. cinereus*) group average distance from *M. avidus* is 1.03 ± 0.13 (III)

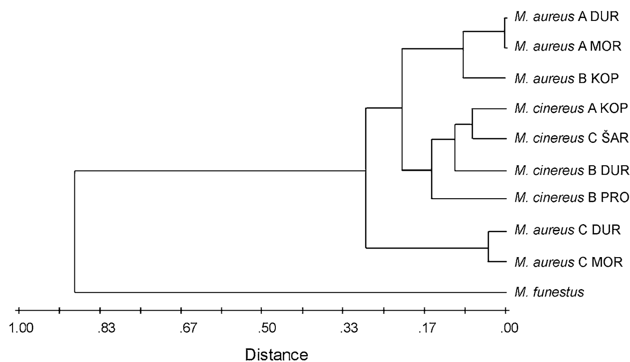

Fig. 4 UPGMA dendrogram (based on Unbiased Genetic Distance; Nei 1978) generated using allozyme data for 10 populations of seven species of the *M. aureus* group and *M. funestus*. Goodness of fit statistics: *f* (Farris 1972) = 2.12; *F* (Prager and Wilson 1976) = 13.79; % standard deviation (Fitch and Margoliash 1967) = 29.07; Cophenetic correlation = 0.98

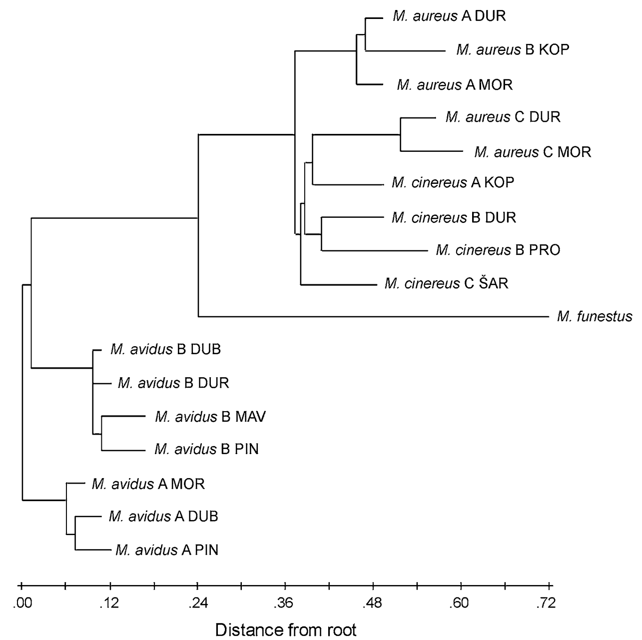

Fig. 5 Wagner procedure dendrogram using Prevosti's distance (Wright 1978) among 10 populations of the *M. aureus* group, *M. funestus* and seven populations of the *M. avidus* outgroup generated using allozyme data. Tree rooted using outgroup method after optimization. Goodness of fit statistics: *f* (Farris 1972) = 2.79; *F* (Prager and Wilson 1976) = 4.19; % standard deviation (Fitch and Margoliash 1967) = 12.76; Cophenetic correlation = 0.99

and 17.20% of mutations were at the first position. Out of a total of 93 variable sites, 16 were non-synonymous, and all of these occurred at the first-codon position.

The parsimony analysis of 23 ingroup terminals of the *aureus–cinereus* complex using *Merodon avidus* A species as outgroup (including sequence data of three additional *M. avidus* specimens, two *M. avidus* B and one *M. avidus* A) resulted in one parsimonious tree with a length of 158 steps CI = 0.87, RI = 0.96 (Fig. 2). The resulting tree had two main clades, (*M. aureus* A + *M. aureus* B) and ((*M. aureus* C + *M. cinereus* B + *M. cinereus* C) + *M. cinereus* A), that differed on average by 5.74% (uncorrected pairwise divergence).

Discussion

Delimitation of genetic units

The present concept of *M. aureus* comprises three taxa: *M. aureus* A (haplotypes I and II, alleles at *Had, Sod*-1), *M. aureus* B (haplotype III, Pgm^c allele) and *M. aureus* C (haplotypes IV–VII, alleles at *Aat, Had, Me* and *Sod*-1) (Fig. 2; Table 3). Populations that fit the present concept of *Merodon cinereus* are *M. cinereus* A (haplotype VIII, alleles at *Me* locus), *M. cinereus* B (haplotype VII, although species-specific alleles suggest that *M. cinereus* B DUR and *M. cinereus* B PRO populations are likely at an early stage of speciation, in the absence of differences at mtDNA and morphological traits they are considered as conspecific for the time being) and *M. cinereus* C (haplotype VII, alleles at *Me* locus). However, the variability of morphological, allozyme and mtDNA data presented herein supports the notion that in delineation of the species there can be no standard level of divergence (Lipscomb et al. 2003; Will and Rubinoff 2004), and more than one source of characters should be considered in species delimitation.

The current study highlights the importance of integrating molecular and morphological characters (e.g.

Table 5 Raw fixed differences (lower matrix) and Uncorrected ("*p*") distance matrix for haplotypes of the *M. aureus, M. cinereus* complexes and *M. funestus* (I: *M. aureus* A MOR, *M. aureus* A DUR; II: *M. aureus* A MOR; III: *M. aureus* B KOP; IV: *M. aureus* C DUR; V: *M. aureus* C DUR; VI: *M. aureus* C MOR; VII: *M. aureus* C MOR, *M. cinereus* C ŠAR, *M. cinereus* B DUR, *M. cinereus* B PRO; VIII: *M. cinereus* A KOP; IX: *M. funestus* MOR; X: *M. funestus* MOR)

Haplotype	I	II	III	IV	V	VI	VII	VIII	IX	X
I	–	0.14	1.12	5.77	5.49	5.91	5.77	6.03	7.46	7.61
II	1	–	1.27	5.91	5.63	6.06	5.91	6.18	7.32	7.46
III	8	9	–	5.49	5.21	5.63	5.59	5.45	7.75	7.89
IV	42	42	40	–	0.29	0.84	0.28	0.71	9.58	9.72
V	41	41	39	1	–	1.13	0.57	1.01	9.58	9.72
VI	43	44	41	6	7	–	0.85	0.86	9.72	9.86
VII	42	43	40	2	3	6	–	0.43	9.58	9.72
VIII	42	43	38	6	7	7	4	–	9.42	9.57
IX	53	52	55	69	70	70	69	65	–	0.42
X	54	53	56	70	71	71	70	66	3	–

Moritz and Cicero 2004; Rubinoff and Holland 2005; Rubinoff 2006), especially when dealing with closely related species, recently diverged taxa and taxa in the progress of divergence and speciation (Avise 2000; Funk and Omland 2003). Both allozymes (diagnostic loci and a species-specific allele) and COI mtDNA (unique haplotypes) allowed determining species boundaries for morphologically inseparable *M. aureus* A and *M. aureus* B. However, while species-specific allelomorphs clearly delineated cryptic taxa within the morphologically defined *M. aureus* and *M. cinereus* species, the allozyme data were not informative enough to conclusively resolve the status of the spring and summer generations of *M. funestus.* On the other hand, morphological and mtDNA COI markers failed to discriminate some reproductively isolated but recently separated sibling taxa although they were clearly defined by allozyme markers. Relatively high gene flow might have prevented differentiation at loci not experiencing selection, which may explain low variability in morphology and sequenced mtDNA. High similarity of morphological traits in cryptic or unrecognized species is often a result of multiple factors, such as retention of ancestral morphology and convergent morphological evolution due to similar selection pressures (cf. Funk and Omland 2003). Low mtDNA diversity could reflect both recent common ancestry and stabilizing selection, or small ancestral effective population size (Hedrick et al. 2006). Additionally, longer coalescence time for mtDNA than for nuclear alleles could be results of the existence of polygynous mating systems and sex-biased dispersal (female philopatry) (Hoelzer 1997). Furthermore, introgression following hybridization and paralogy resulting from transfer of mtDNA gene copies to nucleus might limit the utility of mtDNA in recognition of species boundaries (Moritz and Cicero 2004).

Genetic and phylogenetic relationships

The observed spatial distribution of the taxonomic diversity of the *aureus* group suggests that the group is of Mediterranean ancestry, while relatively low differentiation among taxa indicates that lineages are at an early stage of divergence. As the larvae are internal feeders in bulbs and rhizomes (Roteray 1993), the relationship of *Merodon* species with bulb plants could be one of the main drivers of diversity and distribution of the genus. Like the *Merodon* genus, the center of diversity of bulb plants is the Mediterranean region (Mensel et al. 1965). One possible mechanism of diversification within the *aureus* group might be the fragmentation of a widespread ancestor and divergence in multiple glacial refugia during the Pleistocene. Another mechanism of diversification of the *aureus* and *cinereus* complexes might include founder events during recolonization of previously glaciated areas, and drift among allopatric, refugial populations. The influence of drift is supported by the very low mtDNA haplotype diversity. Using a conservative calibration factor of 1 D = 5 Myr (Nei 1987) the earliest divergence was observed for the species pair *M. aureus* B and *M. aureus* C (1.76 Ma), while *M. cinereus* A and *M. cinereus* C appear to be the most closely related species (time of divergence estimated at 0.33 Ma).

Sequencing data of two specimens of the *M. cinereus* complex from Pelister (FYR Macedonia) (Ståhls unpublished) showed a unique haplotype in a phylogeographically distant population and its closer relationships to geographically close species *M. cinereus* C and *M. cinereus* B populations than to geographically distant *M. cinereus* A. Taking into account that younger lineages typically occupy more restricted ranges than older lineages (Neigel and Avise

1993), *M. aureus* B and populations within the *M. cinereus* A/*M. cinereus* C lineage seem to have diverged into genetically distinct gene pools only recently. Comparing mtDNA sequences from our study with the sequences of the members of the *aureus* group, *M. aureus* B was most closely related to the morphologically defined *M. aureus* species from Germany, with only one variable nucleotide site (Ståhls personal observation). Furthermore, based on the preliminary data, *M. unicolor* Strobl (Spain) is genetically the closest species to *M. aureus* B (Vujić et al. personal observation). These results indicated genetic diversity and radiation within *M. aureus* B lineage. We expect that the ongoing research will give us better insight into evolutionary relationships among spatially fragmented units of *M. aureus* B.

The UPGMA, Wagner and parsimony analyses presented a different pattern of the genetic relationship among members of the *aureus* group, which is not surprising considering that Wagner method and phenetic clustering differ in their algorithms and assumptions, and taking into account that nuclear and mitochondrial genomes evolve at different rates. In all runs the species pair *M. aureus* A/*M. aureus* B was placed as a monophyletic group and *M. funestus* was the most distant species. However, placements of the members of *M. aureus* C and taxa of the *cinereus* complex were different, reflecting both different diversity of allozyme loci and mt gene and different pattern of evolutionary changes. In all analyses a clearly defined lineage was the *M. cinereus* A taxon, indicating independent evolution of this species. The *M. aureus* C, *M. cinereus* B and *M. cinereus* C taxa were well defined by allozyme markers. Despite the lack of the morphological divergence between *M. cinereus* A and *M. cinereus* C, allozyme loci and mtDNA showed clear divergence. In the UPGMA analysis, *M. cinereus* A and *M. cinereus* C were monophyletic, which was inconsistent with mtDNA parsimony analysis and Wagner method. On the other hand, the relationship between the two populations of *M. cinereus* B depended on DNA markers used. In spite of diagnostic alleles at the *Gpi* locus and differences in allelic frequencies, these populations shared the same COI haplotype (haplotype VII), which was likely a plesiomorph character.

Conservation implications

Based on the observed private alleles and genotypes, unique heterozygotes, significant differences of allelic frequencies and recognized cryptic species across the full hierarchy of taxonomic and genetic diversity, it seems that allelic diversity is a suitable representation of the evolutionary potential in the *aureus* group, as is the case in the *ruficornis* (Milankov et al. in press) and *avidus* (Milankov et al. 2001) groups of the *Merodon* genus. High level of genetic variation within analyzed populations of the *M. aureus* group compared to other hoverflies (e.g. Milankov et al. 2005; Ludoški et al. 2004) indicates large evolutionary potential that should be addressed through conservation management. Here we have obtained results that some loci had higher values of genetic differentiation, and we hypothesized that selection might have played a major role in creating genetic divergence among populations (under selective neutrality, all loci would be similarly affected by gene flow, historical effects and genetic drift). Observed higher level of allelic diversity at allozyme loci than in mtDNA COI gene could also be evidence of the influence of selection on the loci that are likely to be under selection or tightly linked loci. Finally, the existence of specific alleles in allochronic and sympatric populations (*M. funestus* MOR) points to influence of different environments driving genetic divergence among generations, and may be a sign of the intraspecific differentiation above the level of allochronic conspecific populations.

Using the available morphological and genetic data of the *Merodon* taxa, the present study partially resolved the taxonomic status and evolutionary relationships within the genus, and defined effective conservation units. Bearing in mind that those populations that became isolated in the remaining habitat face a risk of extinction (Bolger et al. 1997), protection of phylogenetic lineages within the sensitive *aureus* taxa and conservation of detected genetic variation are essential. Most of the localities of the analyzed populations are within already protected areas, such as the National Parks Durmitor, Kopaonik, Šar planina Mts. and Pindos. However, Prokletije Mt and Morinj locality have not been protected. Because of the extraordinary biodiversity, observed endemic and unique genetic diversity, Morinj locality urgently needs to be incorporated into conservation management planning. Morinj is the most threatened locality among all mentioned, due to the high degree of human encroachment, small size of diverse ecosystem patches, and small populations of many rare and endangered hoverflies species (Vujić et al. personal observation). This area was proposed for protection in 2001 but still no decision has been reached.

Acknowledgements The authors wish to thank Jasmina Ludoski for her help with the technical matters. This work was supported in part by the Ministry of Science and Environmental Protection of Serbia, Grant Number 143006B, the Provincial Secretariat for Science and Technological Development (Maintenance of biodiversity – “Hot spots” on the Balkan and Iberian Peninsula) and the Carl Cedercreutz Foundation (Helsinki, Finland).

References

Avise JC (2000) Phylogeography: the history and formation of species. Harvard University Press, Cambridge, MA

Ayala FJ, Powell JR (1972) Allozymes as diagnostic characters of sibling species of *Drosophila*. Proc Natl Acad Sci USA 69(5):1094–1096

Bolger DT, Alberts AC, Sauvajot RM et al (1997) Response of rodents to habitat fragmentation in costal southern California. Ecol Appl 7:552–563

Clary D, Wolstenholme D (1985) The mitochondrial DNA molecule of *Drosophila yakuba*: nucleotide sequence, gene organization, and genetic code. J Mol Evol 22:252–271

Farris JS (1972) Estimating phylogenetic trees from distance matrices. Am Nat 106:645–668

Fitch WM, Margoliash E (1967) Construction of phylogenetic trees. Science 155:279–284

Funk JD, Omland KE (2003) Species-level paraphyly and polyphyly: frequency, causes and consequences, with insights from animal mitochondrial DNA. Annu Rev Ecol Evol Syst 34:397–423

Goloboff P (1999) Analyzing large data sets in reasonable times: solutions for composite optima. Cladistics 15:415–428

Hedrick PW, Lee RN, Hurt CR (2006) The endangered Sonoran topminnow: examination of species and ESUs using three mtDNA genes. Conserv Genet 7:483–492

Hoelzer GA (1997) Inferring phylogenies from mtDNA variation: mitochondrial-gene trees versus nuclear-gene trees revisited. Evolution 51:622–626

Levene H (1949) On a matching problem arising in genetics. Ann Math Stat 20:91–94

Lipscomb D, Platnick N, Wheeler Q (2003) The intellectual content of taxonomy: a comment on DNA taxonomy. Trends Ecol Evol 18:65–66

Ludoški J, Milankov V, Vujić A (2004) Low genetic differentiation among conspecific populations of Melanogaster nuda (Diptera, Syrphidae). Int J Dipterol Res 15:229–235

Marcos-Garcia M[a]A, Vujić A, Mengual X (2007) Revision of Iberian species of the genus *Merodon* Meigen, 1803 (Diptera: Syrphidae). Eur J Entomol 104:531–572

Mensel H, Jäger E, Weinert E (1965) Vergleichende chorologie der zentraleuropäischen flora I, text. Veb Gustav Fisher Verlag, Jena

Milankov V (2001) Evolutionary relationships of the *ruficornis* and *aeneus* groups of species of the genus *Merodon* Meigein, 1803 (Diptera: Syrphidae). Ph.D. Thesis, University of Novi Sad (in Serbian, English abstr.)

Milankov V, Ståhls G, Vujić A (in press) Molecular diversity of populations of the *Merodon ruficornis* group (Diptera, Syrphidae) on the Balkan Peninsula. J Zool Syst Evol Res

Milankov V, Stamenković J, Ludoški J, Ståhls G, Vujić A (2005) Diagnostic molecular markers and the genetic relationships among tree species of the *Cheilosia canicularis* group (Diptera: Syrphidae). Eur J Entomol 102:125–131

Milankov V, Vujić A, Ludoški J (2001) Genetic divergence among cryptic taxa of *Merodon avidus* (Rossi, 1790) (Diptera: Syrphidae). Int J Dipterol Res 12:15–24

Moritz C (1994) Defining "Evolutionary Significant Units" for conservation. Trends Ecol Evol 9:373–375

Moritz C, Cicero C (2004) DNA barcoding: promis and pitfalls. PloS Biol 2:1529–1531

Munstermann LE (1979) Isozymes of *Aedes aegypti*: phenotypes, linkage, and use of genetic analysis of sympatric population in East Africa. D. Phil. Thesis, University of Notre Dame, Indiana

Nei M (1978) Estimation of average heterozygosity and genetic distance from a small number of individuals. Genetics 89:583–590

Nei M (1987) Molecular evolutionary genetics. Columbia University Press, New York

Neigel JE, Avise JC (1993) Application of a random walk model to geographic distributions of animal mitochondrial DNA variation. Genetics 135:1209–1220

Nixon KC (2002) *WinClada* version 1.00.08, Published by author, Ithaca, New York (http://www.cladistics.com)

Pasteur N, Pasteur G, Bonhomme F, Catalan J, Britton-Davidian J (1988) Practical isozyme genetics. Ellis Horwood Limited, Chichester

Peck LV (1988) Family Syrphidae. In: Soos A (ed) Catalogue of Palaearctic Diptera, vol 8. Akademia Kiado, Budapest, pp 11–230

Prager EM, Wilson AC (1976) Congruency of phylogenies derived from different proteins. J Mol Evol 9:45–57

Rojo S, Ståhls G, Pérez-Bañón C, Marcos-García MA (2006) Larval morphology and taxonomic implications based on mitochondrial DNA sequences of West Palaearctic *tibialis*-group species of subgenus *Pandasyopthalmus* (Diptera: Syrphidae: *Paragus*). Eur J Entomol 103:443–458

Roteray G (1993) Colour guide to heverfly larvae (Diptera: Syrphidae). Dipt Digest 9:1–156

Rubinoff D (2006) Utility of mitochondrial DNA barcodes in species conservation. Conserv Biol 20(4):1026–1033

Rubinoff D, Holland BS (2005) Between two extremes: mitochondrial DNA is neither the panacea nor the nemesis of phylogenetic and taxonomic inference. Syst Biol 54:952–961

Simon C, Frati F, Beckenbach A, Crespi B, Liu H, Flook P (1994) Evolution, weighing, and phylogenetic utility of mitochondrial gene sequences and a compilation of conserved polymerase chain reaction primers. Ann Entomol Soc Am 87:651–701

Sneath PHA, Sokal RR (1973) Numerical taxonomy. W.H. Freeman and Company, San Francisco

Speight MCD (2004) Species accounts of European Syrphidae (Diptera) 2004. In: Speight MCD, Castella E, Sarthou J.-P, Monteil C (eds) Syrph the Net, the database of European Syrphidae, vol 44. Syrph the Net publications, Dublin

Swofford DL, Selander RB (1989) BIOSYS-1: a computer program for the analysis of allelic variation in population genetics and biochemical systematics. Release 1.7. Users manual. Illinois Natural History Survey, Champaign, pp 1–42

Šimić S, Vujić A (1996) Hoverfly fauna (Diptera: Syrphidae) of the southern part of the mountain Stara Planina, Serbia. Acta ent serb 1:21–30

Thompson FC (ed) (2005) Biosystematic Database of World Diptera. http://www.diptera.org/names, accessed on 19 January 2006

Thorpe JP (1982) The molecular clock hypothesis: Biochemical evaluation, genetic differentiation and systematics. Annu Rev Ecol Syst 13:139–168

Van de Wweyer G, Dils J (2002) Contribution to the knowledge of the Syrphidae from Greece (Diptera: Syrphidae). Phegea 27:69–77

Weir BS (1996) Genetic data analysis II. Sinauer Associates, Sunderland, MA

Will KW, Rubinoff D (2004) Myth of the molecule: DNA barcodes for species cannot replace morphology for identification and classification. Cladistics 20:47–55

Wright S (1978) Evolution and the genetics of populations, vol 4. Variability within and among natural populations. University of Chicago Press, Chicago