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Usage of Different Molecular Markers in Delimitation of Cryptic Taxa in *Merodon avidus* Species Complex (Diptera: Syrphidae)

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Abstract: Three populations of *Merodon avidus* species complex from two geographical areas in the Danube River region (Djerdap and Fruska Gora) were used in this study with the aim to delimitate cryptic taxa within the complex. According to the diagnostic morphological characters, as well as to the season of their adult activity, samples were presumably identified as *M. avidus* Rossi or *M. moenium* Wiedemann. In order to reveal genetic differentiation between these taxa and to evaluate their current taxonomic status, two different types of molecular markers were used, allozymes and mtDNA. The analysis of 5 enzyme loci revealed the clear presence of two separate taxa, with presence of unique alleles in AAT (EC number 2.6.1.1) and ME (EC number 1.1.1.40) loci in *M. avidus* and *M. moenium* populations. The UPGMA dendogram based on Nei's genetic distance showed the presence of *M. avidus* and *M. moenium* cluster. On the other hand, the parsimony tree based on cytochrome c oxidase subunit I (COI) mtDNA sequences failed to discriminate these two taxa. The haplotype analysis revealed that one haplotype was shared between *M. avidus* and *M. moenium*. This led us to the conclusion that the allozyme data with species-specific alleles are more informative for resolving taxonomic questions in *M. avidus* species complex compared to the mtDNA marker. Moreover, the allozymes confirmed different seasonal preferences of the two taxa when they exist at the same locality. Since the studies of taxonomically diverse and challenging taxa are important for identification of areas of genetic endemism, we highly recommend integrative usage of allozyme and morphological markers.

Keywords: Allozyme, cryptic taxa, COI, *Merodon avidus,* Syrphidae

Introduction

The genus *Merodon* (Meigen, 1803) is the largest Europaean genus of hoverflies family (Vujic *et al*. 2012) and hence it represents an informative source for characterisation of biological diversity of Syrphidae. Studying its genetic structure and phylogenetic relationships is challenging because of the high diversity, presence of cryptic species, taxonomic ambiguities and unclear relationship among the taxa. The high taxonomic and genetic diversity of hoverflies is noticeable on the Balkan Peninsula, which is likely due to biogeographical history during the Pleistocene, when this area was an important refugium for plants and animals (Hewitt 2000).

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Merodon avidus (Rossi, 1790) is an extremely widespread European species, being absent only in northern areas of Europe, and which is also present in North Africa, the Middle East, and Asia Minor (Dirickx 1994). In spite of some defined species-specific morphological signs, Hurkmans (1993) pointed that the great variation in the colouration of the antennae, thorax, legs, and abdomen is the probable cause of taxonomic difficulties in the correct identification of certain specimens. This author highlighted that no distinguishable geographic trend in morphology or colour of the various local forms had been found and the intrapopulation variability in size and colour was explained as a consequence of the quantity and quality of food ingested during the larval stages. Almost a decade later, the analysis of 5 morphological features of the tergite II and III, tibiae and mesoscutum, as well as the allozyme variability analysis confirmed that *M. avidus* is actually a separate geographically and genetically structured taxon, comprising a diverse group of cryptic taxa (Milankov *et al.* 2001). This indicated that *M. avidus* represents a complex of cryptic species, which are likely to be delimitated using suggested diagnostic characters (morphological and biochemical).

As previously demonstrated in hoverflies (Schönrogge *et al*. 2002), the detection of cryptic speciation could be an important step in conservation planning, as well as in natural resource protection and management. Inability to identify biologically important species therefore hampers our efforts to conserve, study or utilise them (Bickford *et al.* 2007).

The aim of this study was to delimitate cryptic taxa within *Merodon avidus* species complex and to quantify genetic variability of the natural Danube River populations. In order to reveal genetic differentiation among these taxa and to evaluate their current taxonomic status, two different types of molecular markers were used, allozymes and mtDNA. The final purpose of this study was to compare the benefits of these markers in revealing potential species boundaries between the populations.

Material and Methods

Sample collection

The identification of specimens was based on studying type material from several museums and was made according to certain diagnostic morphological characters (Milankov *et al.* 2001), as well as to the season of their adult activity (spring season V-VII, autumn season VIII-IX). For the first time we decided to use the following names for two distinct taxa: *Merodon avidus* (Rossi, 1790) and *Merodon moenium* (Wiedemann, 1822), on the grounds of a recent taxonomic revision (Vujić *et al*. in prep.). The samples were collected from two different Danube River regions in Serbia: Djerdap - gorge and Fruska Gora - low mountain. The sampling in Djerdap was done during the spring season (mean daily temperature 19.5°C) and during the autumn season (mean daily temperature 20.2°C). The specimens from Fruska Gora were collected during the spring season (mean daily temperature 18.9°C). A total of 21 insects were used for allozyme analysis (Djerdap, June specimens - presumably identified as *M. moenium* and September speciemens - presumably identified as *M. avidus;* Fruska Gora, July specimens - presumably identified as *M. moenium*). The COI analysis included 23 insects (Djerdap, June specimens - presumably identified as *M. moenium* and September specimens - presumably identified as *M. avidus*; Fruska Gora, June specimens presumably identified as *M. moenium*).

Allozyme analysis

The genetic variation of allozymes was studied by standard 5% polyacrylamide gel electrophoresis (Munstermann 1979) with slight modifications. A set of five previously established diagnostic enzymes for *Merodon avidus* species complex (Milankov *et al.* 2001) was selected for this analysis: malic enzyme (ME, 1.1.1.40), isocitrate dehydrogenase (IDH, 1.1.1.42; *Idh-2*)*,* α-glycerophosphate dehydrogenase (GPD, 1.1.1.8; *Gpd-2*), aspartate amino transferase (AAT; 2.6.1.1), and glucosephosphate isomerase (GPI, EC 5.3.1.9). Genotype and allele frequencies were calculated directly from the observed banding patterns based on the genetic interpretation of zymograms. The statistical analysis of the allozyme data was performed using the computer program POPGENE Version 1.32 (Yeh *et al*. 1999).

Cytochrome oxidase c subunit I (COI) sequence analysis

DNA was extracted from legs and abdomen of the speciemens using SDS Extraction Protocol (Chen *et al*. 2010) with slight modifications. Subsequently,

the cytochrome c oxidase subunit (COI) of mitochondrial DNA was amplified. PCR reactions were carried out in 20 µl reaction volume using primers C1-J-2183 (5'-CAA CAT TTA TTT TGA TTT TTT GG-3') (alias JERRY) i TL2-N-3014 (5'-TCC AAT GCA CTA ATC TGC CAT ATT A-3') (alias PAT) (Simon *et al*. 1994). The reaction mix consisted of 1x PCR buffer, 2.5 mM MgCl2, 0.1 mM of each nucleotide, 1 U Taq polymerase, 2 pmol of each primer, and 50 ng DNK. The mtDNA COI region was amplified using an Eppendorf Personal Thermocycler (initial denaturation for 2 min at 95°C; 30 s denaturation at 94°, 30 s annealing at 49°C, 2 min extension at 72°C/30 cycles; and the final extension for 8 min at 72°C). The PCR products were purified by the Exo-Sap purification method, according to a protocol of the manufacturer (Fermentas). The 667-bp fragments of COI gene were sequenced in forward direction. The number of haplotypes and haplotype diversity (Hd) were defined using DnaSP v5 (Librado, Rozas 2009). The parsimony analysis of the available sequences 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 TBR branch swapping. All base positions were treated as equally weighted characters.

Results and Discussion

During the years of the hoverfly collecting seasons our research group noticed that *M. moenium* specimens generally inhabited areas with lower average temperatures compared to *M. avidus* specimens. However, habitats in which these populations existed in sympatry were also found (*e.g.* Djerdap). For these kind of localities it was suspected that activity of particular adult population was determined by seasonal changes - *M. moenium* populations seemed to appear after a cold period (winter) and exist during the season with lower average temperature than *M. avidus* populations, whose adults appeared later. This was supported by the fact that after July no *M. moenium* specimens could be found at any locality. According to that fact, in this research it was assumed that the spring generation in Djerdap represented *M. moenium* taxa, while the autumn generation represented *M. avidus*. This was also in concordance with the observed diagnostic morphological signs and the "separation by season" hypothesis, which was intended to be clarified in this paper, using molecular markers.

Three of the five analysed enzyme loci were polymorphic in all populations of *M. avidus* complex (GPD, IDH and GPI). On the other hand, AAT locus showed the presence of one common allele (a) in all populations that were initially identified as *M. avidus*, and the presence of other common allele (b) in all populations that were identified as *M. moenium* taxon. This indicated that AAT locus can be considered completely diagnostic for delimitation of cryptic taxa, as was previously suggested (Milankov *et al*. 2001). Furthermore, ME locus was monomorphic within *M. avidus* population (fixed "b" allele) and within *M. moenium* population (fixed "a" allele) in this study. According to the data obtained from the analysed region, ME can be considered as diagnostic for separating *M. avidus* and *M. moenium* taxa.

The hierarchical structure of *M. avidus* species complex was analysed using the main parameters of F statistics for *M. avidus* total population divided into two subpopulations (*M. avidus* and *M. moenium*) (Table 1). The presence of high genetic differentation among the *M. avidus* and *M. moenium* populations was detected (Fst =0.515). F_{is} showed positive value at IDH locus, suggesting an excess of homozigosity. Nevertheless, $F_{st} > F_{is}$ relation at each locus, including IDH, suggested that genetic drift and not local inbreeding affected this result. Considering that the effect of genetic drift, historical effect, and gene flow are almost equal at all loci, similar F_{st} in all polymorphic loci could be expected. However, different F_{st} values across loci in this study (Table 1), indicated that genetic drift and gene flow had not been major factors in the observed genetic divergence between the analysed populations. This suggested that different selection pressures constituted the most important force in creating genetic differenatiation between *M. avidus* and *M. moenium* populations, not only in different geographical areas, but also within the same habitat (Djerdap). We might suppose that some abiotic factor(s) may result in creating genetic variability between two cryptic taxa. The general temperature differences between the distinct habitats of *M. avidus* and *M. moenium*, as well as the average seasonal differences in their shared habitats suggested that temperature could possibly act as the most important selective factor. This factor was already mentioned as a frequent

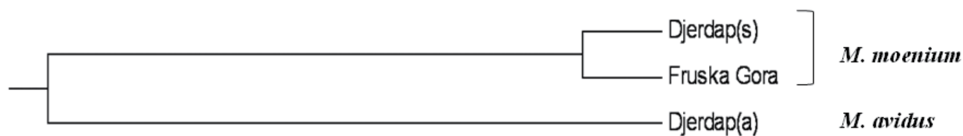

Fig. 1. Dendogram of populations of *M. avidus* species complex based on Nei's (1978) genetic distance (Method = UPGMA, Modified from NEIGHBOR procedure of PHYLIP Version 3.5). (a) – autumn collecting season, (s) – spring collecting season

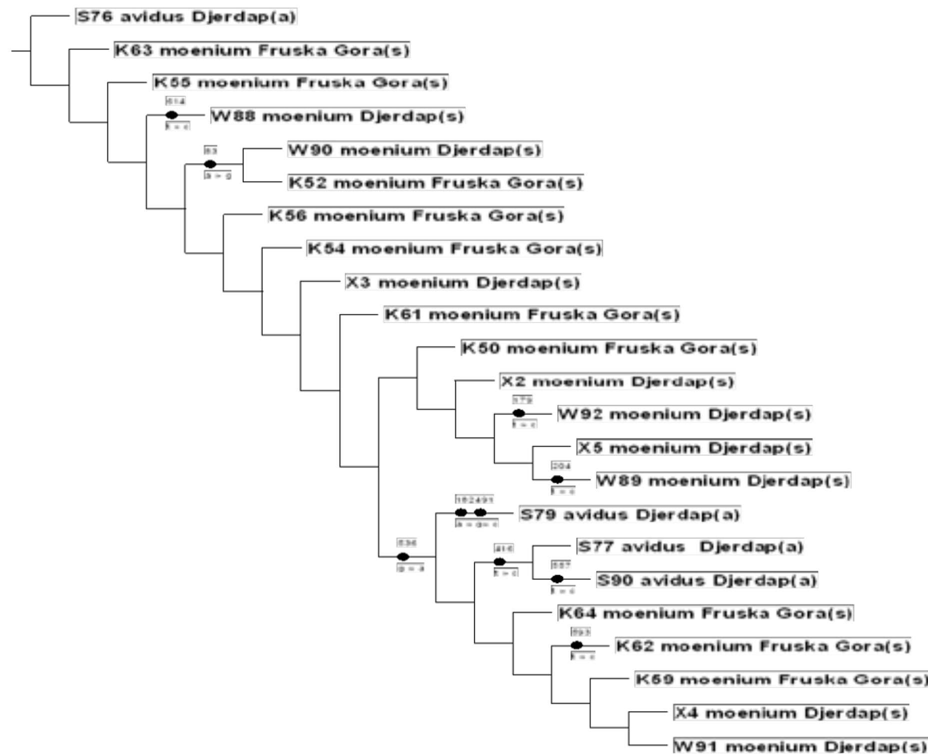

Fig. 2. The most parsimonious tree based on mitochondrial data, length =10, CI=100, RI=100. Filled circles = nonhomoplasious changes. (a) – autumn collecting season, (s) – spring collecting season

cause of gradual changes in allozyme frequencies in the poikilotherms, whose metabolism is to a great extent modified by the temperature changes in the environment, *e.g.* at G-6-PDH in killifish, *Fundulus heteroclitus* (Powers, Place 1978), and at *Est-I, Xdh, Ao* and *Alb* loci in aquatic marsh frogs, *Pelophylax ridibundus* (Nevo, Yang 1982). Since the observed differences in our F_{st} values encourage the hypothesis about temperature as an enviromental factor of selection, the future examination of enviromental factors would be of great importance in establishing the main cause of the perceived genetic differentiation between these taxa.

The analysis of the allele distribution was quantified by unbiased measures of genetic identity and genetic distance (Nei 1978). The averaged value of genetic identity (0.293) betweeen *M. avidus* and *M. moenium* was much lower than averaged value of genetic distance (1.227) between these populations. In addition, the dendogram based on Nei's (1978) genetic distance (UPGMA method) confirmed the presence of two monophyletic groups: *M. avidus* cluster and *M. moenium* cluster (Fig. 1).

The COI analysis of 23 speciemens determined the presence of ten haplotypes. Three unique haplotypes corresponded to *M. avidus*, six to *M. moenium*, while one haplotype was shared by *M. avidus* and *M. moenium*. In spite of the high haplotype diversity value (Hd=0.795), the presence of shared haplotype limited our ability to definitely separate the two cryptic taxa. The parsimony analysis of 23 specimens from three populations of *M. avidus* species complex resulted in one parsimonious tree with a length of 10 steps CI=100, RI=100 (Fig. 2). Unlike the UPGMA dendogram based on allozyme data, the position of branches of the resulting tree failed to discriminate

Table 1. F statistics parameters in populations of *M. avidus* species complex

Subject of analysis	***M. avidus* and *M. moenium* (2 subpopulations)**		
Locus	F_{IS}	F_{IT}	F_{ST}
GPD	-0.571	-0.545	0.017
IDH	0.657	0.904	0.721
AAT	-	1.000	1.000
GPI	-0.765	-0.714	0.029
ME	-	1.000	1.000
Mean	-0.507	0.269	0.515

the two cryptic taxa: *M. avidus* and *M. moenium*. This led us to conclusion that the allozyme data with species-specific alleles are more informative for resolving taxonomic questions in *M. avidus* species complex compared to the COI mtDNA marker.

Conclusions

Keeping in mind that discovery of geographical and habitat-related patterns in distribution of cryptic species may lead to reconsideration the conservation of particular habitats, it is clear that the identification and description of effective conservaton units have important implications for natural resource protection and management (Bickford *et al*. 2007). In this study, the integrative usage of morphological data and allozyme markers confirmed the presence of two cryptic taxa in *Merodon avidus* species complex, while the COI mtDNA markers appeared to be less applicable in this respect. A possible seasonal pattern in delimitation of the two cryptic taxa was also suggested, with high recommendation of testing some key abiotic factors in future. Since a few DNA markers have been available for the genus *Merodon* so far, the development of new, preferably nuclear DNA markers might be an important future step in the detailed describing species boundaries between these populations.

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