Arch Biol Sci. 2017;69(3):553-560 https://doi.org/10.2298/ABS160729131A

Assessment of genetic diversity within the *Merodon ruficornis* species group (Diptera: Syrphidae) by RAPD analysis

Andrijana Andrić[1,*], Nataša Kočiš Tubić[2], Mihajla Đan[2], Ante Vujić[2] and Dragana Obreht Vidaković[2]

[1] *University of Novi Sad, BioSense Institute*, Dr Zorana Đinđića 1, 21000 Novi Sad, Serbia

[2] *University of Novi Sad, Faculty of Sciences, Department of Biology and Ecology*, Trg Dositeja Obradovića 2, 21000 Novi Sad, Serbia

***Corresponding author**: andrijana.andric@dbe.uns.ac.rs

Received: July 29, 2016; **Revised**: November 15, 2016; **Accepted**: December 5, 2016; **Published online**: December 8, 2016

Abstract: As one of the most distinct groups in the hoverfly genus *Merodon*, the monophyletic *ruficornis* species group has been the focus of several studies using different approaches. Molecular methods have shown incongruences between morphological and molecular data. In the present study, we investigated four species of the *Merodon ruficornis* group (i.e. *M. loewi*, *M. armipes*, *M. papillus* and *M. hoplitis*) with the aim of detecting intra- and interspecific genetic diversity, and we examined the usefulness of random amplified polymorphic DNA (RAPD) in an integrative taxonomic approach to species delimitation. Analysis of Nei's genetic variation over all loci showed that genetic diversity for the analyzed *Merodon* species was h=0.24. Based on UPGMA, PCoA and Bayesian clustering analyses, our results clearly differentiated four groups that correspond to the four morphologically-defined *Merodon* species. Among the analyzed species, *M. armipes* and *M. hoplitis* showed the lowest level of genetic divergence; *M. loewi* was clearly separated from both *M. armipes* and *M. papillus*. Based on our data, we propose the use of RAPD-PCR as an additional tool for resolving taxonomic problems within *Merodon*.

Key words: flower flies; genetic diversity; hoverflies; *Merodon*; RAPD

INTRODUCTION

Hoverflies (Diptera: Syrphidae) are one of the most species-rich dipteran families [1]. Among their genera, *Merodon* Meigen, 1803, is comprised of more than 160 species, distributed throughout the Palaearctic and Afrotropical regions [2,3]. This genus has been the subject of numerous studies dealing with its phenotypic diversity as well as its molecular diversity, aimed at providing additional tools for taxon identification and phylogenetic revision [2-4]. Although both the systematics and taxonomy of this genus have recently received attention [2-13], the status of some *Merodon* species still requires clarification. The high diversity and presence of cryptic taxa make the inference of phylogenetic relationships within this genus challenging, especially concerning their evolutionary and conservation biology [9,14,15].

Different *Merodon* species and species complexes have been studied primarily using allozymes and mtDNA cytochrome c oxidase subunit I (COI) sequences (*M. albifrons* Meigen, 1822 [11], the *M. avidus* complex [10,15,16] and the *M. aureus* and *M. cinereus* species complexes [9]). These markers have proven useful in the detection of intra- and interspecific genetic diversity, although results have not always been congruent. Both markers appear to be powerful in integrative taxonomic studies, with an *a priori* advantage of COI for distinguishing species due to its sequence variability. However, allozyme data with species-specific alleles have occasionally proven more informative for species delimitation than mtDNA markers [15]. In addition, the results of certain morphological studies have not always agreed with the results of molecular analyses [5,14].

Discordance between the results of different studies using different approaches to reveal taxonomic boundaries within this genus is especially relevant in the *Merodon ruficornis* species group (*sensu* Milankov et al. 2002 [17] in [13]). Predominantly distributed in the northern and eastern Mediterranean, with the greatest diversity on the Balkan Peninsula, in Turkey

© 2017 by the Serbian Biological Society

and the Caucasus region, this group is characterized by a significantly high level of endemism. Among the 18 recorded species, 12 are known as limited-range endemics, present in small, often isolated parts of the total range of the group [13]. The *M. ruficornis* species group is comprised of closely related Palaearctic taxa that are morphologically extremely similar, with the shape of metalegs being the main diagnostic character in almost all the species [13]. The distinct phylogenetic position of the *ruficornis* species group has been resolved, and molecular and morphological data support its monophyly [13]. The taxonomic boundaries of the several species within the group have been well defined based on wing shape and size. Even interpopulation wing morphometric variability implies high structuring within morphologically clearly defined species, which is in accordance with molecular diversity [5]. However, analyses of mitochondrial and nuclear regions revealed ambiguities regarding interspecies relationships within the *ruficornis* group (i.e. *Merodon loewi* van der Goot, 1964, *M. armipes* Rondani, 1843, *M. papillus* Vujić, Radenković, Pérez-Bañón, 2007) [2,8,13].

The integration of multiple data sources in order to re-evaluate taxonomic species delimitation within the *Merodon* genus is prudent [6,7,11,14-16]. Ever since random amplified polymorphic DNA (RAPD) genetic markers were described [18,19], they have been widely used for the estimation of genetic diversity within and among various insect species and populations [20]. Some of the first applications of such 10-base primers for amplification of random regions of genomic DNA were used to identify subspecies and geographic populations of mosquitoes (Diptera: Culicidae) [21] and to differentiate morphologically indistinguishable cryptic species [22]. Being relatively simple, quick, technically accessible and independent of *a priori* sequence information, RAPD continues to be used successfully in Dipteran studies to the present day, e.g. for determining the population genetic variation of blowflies (Calliphoridae) [23], species characterization of leaf-miner flies (Agromyzidae) [24], and interspecific genetic relationships of fruit flies (Drosophilidae) [25] and houseflies (Muscidae) [26]. Even though they have been shown to be a powerful tool in various molecular analyses of many fly families, to date there are no published data on the application of RAPD markers to Syrphidae.

In the present study, we investigated four *Merodon* species of the *ruficornis* group: *M. loewi, M. armipes, M. papillus* and *M. hoplitis* Hurkmans, 2012 [13]. Since these species are morphologically differentiated and defined, we attempted to resolve a persisting incongruence among the results of analyses based on different molecular markers and morphological characters. The main goal of this study was to detect the genetic diversity of these four species and to clarify their relationships using RAPD markers. Furthermore, we wanted to evaluate the use of RAPD molecular markers as an additional tool for taxon delimitation within *Merodon*.

MATERIALS AND METHODS

Insect material

Genetic diversity was analyzed for 52 specimens of four *Merodon* species belonging to the *ruficornis* group. Sampling was carried out in 2008 and 2010 from April to June. The study area comprised three different localities on the Balkan Peninsula: Lesvos Island (Greece) – *M. papillus* (15 specimens); Đerdap Gorge (Serbia) – *M. loewi* (15 specimens); Orjen Mountain (Montenegro) – *M. armipes* (12 specimens) and *M. hoplitis* (10 specimens). Collection was carried out by hand-net and insects were frozen at -20°C until further use.

DNA extraction and PCR amplification

Total genomic DNA was isolated from 2-3 legs of each specimen following Hondelmann et al. [27]. Genome polymorphism was assessed using a RAPD-PCR marker system. Ten RAPD primers (Operon Technologies Inc., USA) gave clear banding patterns. RAPD-PCR reactions were performed in a volume of 20 μL containing 1 U Taq polymerase (Thermo Scientific), 1×PCR Buffer (Thermo Scientific), 2.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.1 μM of primers and about 100 ng genomic DNA. Amplifications were carried out in an Eppendorf Personal mastercycler in 40 cycles using the following program: initial denaturation at 95°C for 5 min, followed by denaturation at 94°C for 2 min, primer annealing at 36°C for 1 min, elongation at 72°C for 2 min and a final extension at 72°C for 5 min. The amplified products of RAPD-PCR reactions

were separated by horizontal electrophoresis in 1.5% agarose gels, and photographs of the gels were obtained under UV light. Product sizes were determined using a 1 kbp DNA ladder (Serva), and a gene ruler, 100 bp DNA ladder (Thermo Scientific). Clear bands were read (using Adobe Photoshop 7.0) and scored for further statistical analysis.

Data analysis

The binary matrix for further statistical analyses was made according to the DNA banding patterns that were scored for presence (1) or absence (0) of the fragment in each sample. POPGENE ver. 1.32 [28] was used for estimation of genetic variation statistics for all loci [29], i.e. calculation of genetic diversity parameters: h – Nei's [30] gene diversity; I – Shannon's [31] information index, and p – the percentage of polymorphic loci. Arlequin version 3.0 [32] was employed to perform AMOVA (analysis of molecular variance) with 10000 permutations.

Free Tree [33] software was used to calculate genetic similarity among specimens by pairwise comparisons [34]. The resulting coefficients were used to construct a UPGMA (unweighted pair group method with arithmetic mean) dendrogram. Support for the tree topology was assessed by bootstrap analysis using 5000 repetitions, in Free Tree [33]. For graphical representation, data were processed using Tree View [35] software. Principal coordinate analysis (PCoA) was performed using GenAlEx [36] based on the Nei and Li [37] coefficients of genetic distances.

Using STRUCTURE ver. 2.3.3 [38,39], an analysis of population structure based on a Bayesian clustering approach was conducted. Each of the genotype classes was treated as a haploid allele, as recommended by the software documentation, Oliveira et al. [40] and Pinheiro et al. [41]. The admixture ancestry model and the correlated allele frequency model were employed. Runs with a burn-in of 20000 and a Monte Carlo Markov chain (MCMC) of 200000, with 10 repetitions for K=1-10, were set. The results were visualized by CLUMPAK [42]. Using the same software package, an estimate of the most likely number of clusters (K) according to the ΔK method [43] was performed by calculating the log probability of data (lnD(P)) for each value of K [38,44].

RESULTS

RAPD analyses of the four *Merodon* species (52 specimens) with ten different primers resulted in 241 amplified fragments. The number of detected loci varied between primers from 18 (OPA20) to 28 (OPA02) (Table 1). The fragment size ranged from 50 bp to 2 kbp. The total number of polymorphic fragments by species was between 145 (60%) for *M. hoplitis* and 182 (76%) for *M. armipes* (Table 2). The analysis of genetic variation statistics for all loci showed that the Nei's genetic diversity for all analyzed *Merodon* species was h=0.24 and the Shannon index was I=0.39.

Analysis of the distribution of genetic variation among and within species revealed a significantly high variance among the analyzed species (24.53%, $p<10^{-6}$ after Bonferroni correction [45]), despite the high variation detected within species (75.47%) (Table 3).

The UPGMA dendrogram based on Jaccard genetic similarity coefficients showed clear subclustering among different species (Fig. 1). The group consisting of all *M. papillus* specimens formed a cluster that was

Table 1. Attributes of the RAPD primers (length range and number of amplified fragments per primer).

Primer	Nucleotide sequence (5'-3')	Fragment length (Kbp)	Number of fragments
OPA02	5' > TGCCGAGCTG < 3'	0.05-1.55	28
OPA05	5' > AATCGGGCTG < 3'	0.35-2.00	25
OPA07	5' > GAAACGGGTG < 3'	0.30-2.00	25
OPA20	5' > GTTGCGATCC < 3'	0.45-1.45	18
OPB07	5' > GGTGACGCAG < 3'	0.05-1.40	24
OPC04	5' > CCGCATCTAC < 3'	0.30-2.00	25
OPC06	5' > GAACGGACTC < 3'	0.30-2.00	25
OPC10	5' > TGTCTGGGTG < 3'	0.35-2.00	25
OPD05	5' > TGAGCGGACA < 3'	0.05-1.40	27
K15	5' > CTCCTGCCAA < 3'	0.35-1.80	19
Total		0.05-2.00	241

Table 2. Genetic variation statistics per species for all loci.

Merodon species	S	h	I	p (N)	p (%)
M. loewi	15	0.1940	0.3070	174	72.20
M. armipes	12	0.2075	0.3263	182	75.52
M. hoplitis	10	0.1948	0.2973	145	60.17
M. papillus	15	0.1742	0.2788	166	68.88
Total	52	0.2415	0.3874	241	100

S – Sample size (number of analyzed *Merodon* specimens); h – Nei's gene diversity; I – Shannon's Information index; p (N) – number of polymorphic loci; p (%) – percentage of polymorphic loci

Table 3. AMOVA results among and within *Merodon* species.

Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation	*P*-value
Among species	3	498.604	10.41231	24.53	$<10^{-6}$
Within species	48	1538.050	32.04271	75.47	$<10^{-6}$
Fixation index F_{ST} 0.24526					

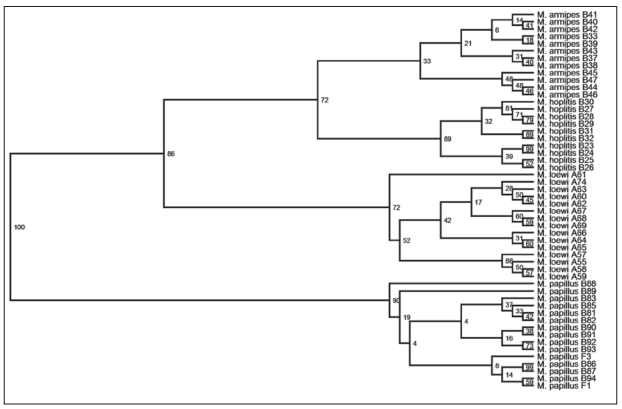

Fig. 1. UPGMA dendrogram of the analyzed *Merodon* specimens based on Jaccard [34] genetic similarity coefficients. Numbers at branches indicate bootstrap values.

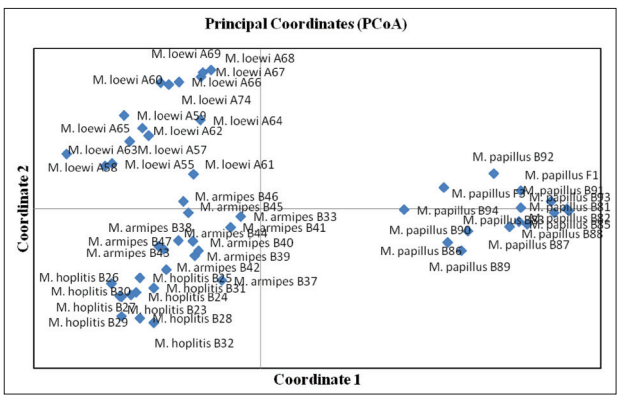

Fig. 2. Principal coordinate analysis based on Nei and Li [37] chord distance among samples. The first and second principal coordinates account for 11.35% and 9.68% of the variation, respectively.

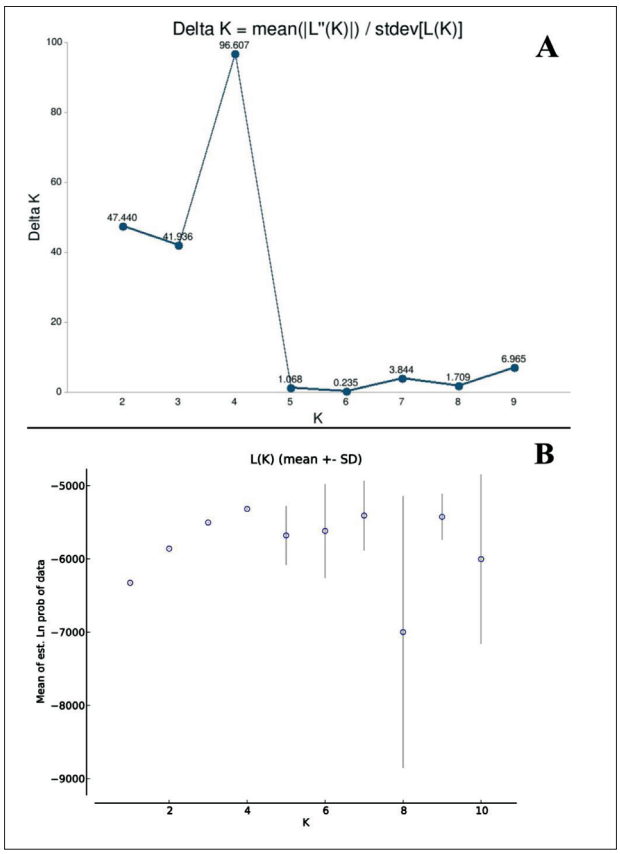

Fig. 3. The most likely number of clusters estimated by: **A** – ΔK method suggesting K=4 and **B** – lnP(D) values supporting K=4.

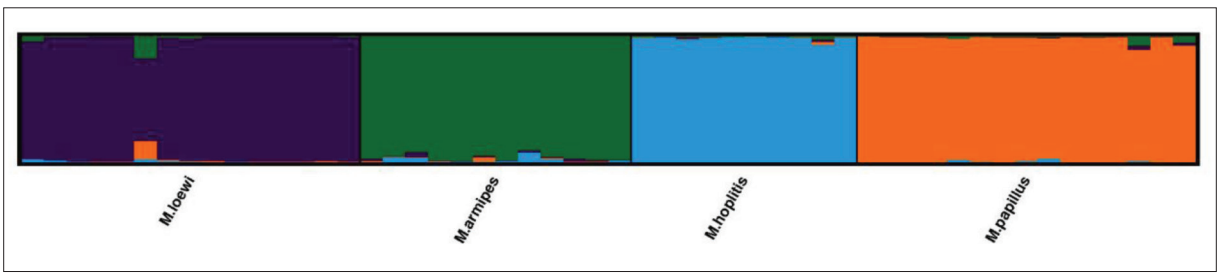

Fig. 4. Population structure of analyzed *Merodon* specimens inferred using the STRUCTURE version 2.3.3 software; cluster partitioning of the individuals at K=4.

separated from the others with high support (bootstrap value of 100). The *M. loewi* group was the next to branch out (86), and *M. hoplitis* and *M. armipes* were resolved as sister groups (with a bootstrap value of 72). A similar grouping of samples was revealed by PCoA (Fig. 2). The first two principal coordinates, explaining a total of 21.02% of the genetic variation, strongly separated samples of *M. papillus* and clearly differentiated *M. loewi*, whereas individuals of *M. hoplitis* and *M. armipes* showed slightly lower genetic divergence from each other.

Results of the Bayesian clustering analysis were in accordance with the previously described results. According to the ΔK method (Fig. 3A) and lnP(D) values (Fig. 3B), four was deemed the most likely number of clusters in the analyzed data. All samples were strongly assigned to one of the four different clusters, clearly differentiating the four analyzed species (Fig. 4).

DISCUSSION

In our RAPD analysis of four *Merodon* species of the *ruficornis* group, the total numbers as well as the percentages of polymorphic fragments per species were the highest for *M. armipes* and the lowest for *M. hoplitis*. Genetic variation per species over all loci revealed the highest values of Nei's diversity and Shannon's index for *M. armipes* and the lowest values for *M. papillus*. Although these genetic diversity parameters did not differ much among the analyzed species, the higher variability in *M. armipes* could be related to the very wide distribution of this species, compared to *M. hoplitis* and *M. papillus* that are both endemic species [13]. It was previously suggested that historic biogeographical processes, as well as selection in different environments, are important factors shaping the taxonomic diversity and population structure of the *M. ruficornis* group [5], and of the *Merodon* genus in general [46]. Analysis of the distribution of genetic variation in our study revealed a relatively high level of genetic differentiation among analyzed species (25%), yet genetic diversity was even higher within each species (75%). Environmental factors interact with microevolutionary processes throughout a species' history, influencing the diversity of fragmented populations [5]. Hence, the high genetic polymorphism detected in this research could reflect changes during the separate evolutionary paths of the studied species.

This was shown to be true especially for *M. papillus* from Lesvos Island, which was the most genetically distinct of the four *Merodon* species examined in our analyses. This result is potentially a consequence of the long-term independent evolution of this endemic species. The importance of geographic isolation in the process of speciation has previously been discussed for the *ruficornis* species group [13]. Despite relatively strong flight ability (i.e. high dispersal potential), *Merodon* hoverfly species are rather sedentary because of the tight connection to the habitats of their larval host plants. The outcome is clear phylogeographic structuring, which has been proven for many *Merodon* species through mtDNA COI barcode haplotype diversity and distribution patterns [46].

In analyzing *Merodon* hoverflies sampled on Lesvos Island by mitochondrial DNA COI barcodes (5'-end of the COI gene), Ståhls et al. [2] successfully distinguished all of the recognized 22 species, except for *M. papillus* and *M. loewi* from the *ruficornis* group. Neither a strict consensus tree (parsimony analyses) nor neighbor-joining cluster analysis succeeded in separating those two species, which were shown to share the same haplotype, despite there being clear morphological differences between them. Since interspecific hybridization is frequently present in similar taxa with geographically overlapping distributions, mitochondrial introgressions and retained ancestral polymorphisms have been suggested as possible reasons for the shared identical haplotypes between these two species [2].

However, in the case of allopatric species, identical COI haplotypes can hardly be the result of introgression. Milankov et al. [8] investigated five species of the *ruficornis* group on the Balkan Peninsula using mtDNA COI sequences (3'-end of the COI gene) and concluded that *M. loewi* and *M. armipes* (from the *ruficornis* group) shared the same COI haplotype, classifying them as a monophyletic group (as revealed by parsimony analysis) even though these taxa are also morphologically well defined. Although both these species have wide distributions [13], *M. loewi* and *M. armipes* inhabit allopatric localities and geographically overlap only to a small extent. Therefore, the shared haplotype of these two species could be the result of

incomplete lineage sorting or stabilizing selection arising from the common ancestor, rather than the result of introgression [8].

In our study, *M. loewi* and *M. armipes* were clearly separated. To our knowledge, this study provides the first DNA evidence for the separation of these species and reveals RAPD as a molecular tool that can be used in integrative taxonomic study of the *Merodon ruficornis* group. Our results are consistent with a UPGMA dendrogram generated from allozyme markers [8]. In addition, in analyzing wing shape and size of *M. armipes* and *M. loewi*, Francuski et al. [5] revealed interspecies phenotypic differentiation between them. There are numerous reasons why delimitation of species boundaries can be limited by single-locus studies, such as those of a mitochondrial gene, including recent radiations or recently and rapidly diverged species. Furthermore, a few sequences are not likely to be representative of the molecular variability of widespread species (such as *M. loewi* and *M. armipes*), and it has previously been suggested that species identifications should be verified using additional gene regions [2]. It is also important to integrate molecular with morphological characters, especially in taxonomic assessments of closely related species and for taxa in the process of divergence and speciation, such as the *M. ruficornis* group [5].

Our RAPD analyses confirmed conclusions based on morphological data, i.e. that *M. loewi* is a clearly differentiated species from *M. armipes* and *M. papillus*. Considering all of the analyzed specimens, our UPGMA, PCoA and particularly the Bayesian clustering analysis clearly partitioned the examined individuals into four groups, which correspond to the four morphologically defined *Merodon* species. Although *M. armipes* and *M. hoplitis*, species previously reported as having clear morphological differences [13], showed the lowest genetic divergence, specimens from both species were assigned to distinct groups, as clearly indicated by our STRUCTURE analysis.

Previous studies that used different molecular markers have not been completely successful in distinguishing some of the morphologically defined species from the *M. ruficornis* group, a highly diverse group with a large proportion of endemic and cryptic species [2,8,13]. Nuclear and mitochondrial genes provide plentiful molecular data as an important information source besides morphological characters, especially in difficult cases of taxa delimitation. However, nuclear and mitochondrial genomes do not share the same inheritance and evolutionary patterns. Different analytical approaches may also result in a lack of consensus between datasets.

RAPD markers have been widely used to characterize insect species, to infer phylogenies and the biogeography of insect populations, and to elucidate modes of evolution [20]. This technique has proven to be a reliable, cost-effective and rapid way to distinguish dipteran species [24], often detecting interspecies differentiation more accurately than other markers (e.g. allozymes) [47]. Applying this method to assess intra- and interspecific genetic relationships, various studies have reached conclusions on the taxonomy and phylogeny of dipteran taxa, emphasizing the importance of an integrative approach [24,25,26,47].

CONCLUSIONS

The present study has shown that RAPD-PCR is a reliable method for examining intra- and interspecific genetic diversity and evaluating taxonomic species delimitation within the *Merodon ruficornis* group. Our results demonstrate the potential of RAPD in population genetic studies and as an additional tool in resolving taxonomic problems within *Merodon*. Considering certain inconsistencies between morphological and molecular data as revealed in previous studies, and the fact that only a few DNA markers have thus far been used for this genus, the development of additional molecular markers is an important step in verifying species delimitation. Taking into account prior research regarding the interspecific relationships of *Merodon*, the results of our study support previous conclusions regarding the need for an integrative taxonomic approach, implying the use of different methods and characters from different sources, i.e. combining molecular, morphological and ecological data.

Acknowledgements: The authors would like to thank Mr John O'Brien for the linguistic revision of the manuscript. This study was supported by the Ministry of Education, Science and Technological Development, Republic of Serbia, Grant No. 173002.

Authors' contribution: Insect material sampling and determination: A.V.; experimental design and laboratory work: D.O.V., M.Đ.; statistical data analysis and interpretation: A.A., N.K.T.; writing the manuscript: A.A., N.K.T.; critical revision of the manuscript: M.Đ., D.O.V., A.V.

Conflict of interest disclosure: The authors report no conflict of interest.

REFERENCES

1. Brown BV, Borkent A, Cumming JM, Wood DM, Woodley NE, Zumbado M. Manual of Central American Diptera, Volume 1. Ottawa: Canadian Science Publishing, NRC Research Press; 2009. 714 p.
2. Ståhls G, Vujić A, Pérez-Bañón C, Radenković S, Rojo S, Petanidou T. COI barcodes for identification of *Merodon* hoverflies (Diptera, Syrphidae) of Lesvos Island, Greece. Mol Ecol Resour. 2009;9(6):1431-8.
3. Vujić A, Marcos-García MÁ, Sarıbıyık S, Ricarte A. New data on the *Merodon* Meigen 1803 fauna (Diptera: Syrphidae) of Turkey including description of a new species and changes in the nomenclatural status of several taxa. Ann Soc Entomol Fr (NS). 2011;47(1-2):78-88.
4. Marcos-García MÁ, Vujić A, Mengual X. Revision of Iberian species of the genus *Merodon* (Diptera: Syrphidae). Eur J Entomol. 2007;104(3):531-72.
5. Francuski Lj, Ludoški J, Vujić A, Milankov V. Wing geometric morphometric inferences on species delimitation and intraspecific divergent units in the *Merodon ruficornis* group (Diptera, Syrphidae) from the Balkan Peninsula. Zool Sci. 2009;26(4):301-8.
6. Marcos-García MÁ, Vujić A, Ricarte A, Ståhls G. Towards an integrated taxonomy of the *Merodon equestris* species complex (Diptera: Syrphidae) including description of a new species, with additional data on Iberian *Merodon*. Can Entomol. 2011;143(4):332-48.
7. Mengual X, Ståhls G, Vujić A, Marcos-García MÁ. Integrative taxonomy of Iberian *Merodon* species (Diptera, Syrphidae). Zootaxa. 2006;1377:1-26.
8. Milankov V, Ståhls G, Vujić A. Molecular diversity of populations of the *Merodon ruficornis* group (Diptera, Syrphidae) on the Balkan Peninsula. J Zool Syst Evol Res. 2008a;46(2):143-52.
9. Milankov V, Ståhls G, Stamenković J, Vujić A. 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. Conserv Genet. 2008b;9(5):1125-37.
10. Milankov V, Ludoški J, Ståhls G, Stamenković J, Vujić A. High molecular and phenotypic diversity in the *Merodon avidus* complex (Diptera, Syrphidae): cryptic speciation in a diverse insect taxon. Zool J Linn Soc - Lond. 2009;155(4):819-33.
11. Milankov V, Ludoški J, Francuski Lj, Ståhls G, Vujić A. Genetic and phenotypic diversity patterns in *Merodon albifrons* Meigen, 1822 (Diptera: Syrphidae): evidence of intraspecific spatial and temporal structuring. Biol J Linn Soc. 2013; 110(2):257-80.
12. Vujić A, Pérez-Bañón C, Radenković S, Ståhls G, Rojo S, Petanidou T, Šimić S. Two new species of the genus *Merodon* Meigen 1803 (Diptera: Syrphidae) from the island of Lesvos (Greece), in the eastern Mediterranean. Ann Soc Entomol Fr (NS). 2007;43(3):319-26.
13. Vujić A, Radenković S, Ståhls G, Ačanski J, Stefanović A, Veselić S, Andrić A, Hayat R. Systematics and taxonomy of the *ruficornis* group of genus *Merodon* Meigen (Diptera: Syrphidae). Syst Entomol. 2012;37(3):578-602.
14. Francuski Lj, Ludoški J, Vujić A, Milankov V. Phenotypic evidence for hidden biodiversity in the *Merodon aureus* group (Diptera, Syrphidae) on the Balkan Peninsula: conservation implication. J Insect Conserv. 2011;15(3):379-88.
15. Popović D, Djan M, Šašić Lj, Šnjegota D, Obreht D, Vujić A. Usage of different molecular markers in delimitation of cryptic taxa in *Merodon avidus* species complex (Diptera: Syrphidae). Acta Zool Bulg. 2014;Suppl7:33-8.
16. Popović D, Ačanski J, Djan M, Obreht D, Vujić A, Radenković S. Sibling species delimitation and nomenclature of the *Merodon avidus* complex (Diptera: Syrphidae). Eur J Entomol. 2015;112(4):790-809.
17. Milankov V, Vujić A, Šimić S. Identifying the species of the *ruficornis* group of the genus *Merodon* Meigen (Diptera: Syrphidae) using morphological and genetic markers. Stud Dipterol. 2002;9:319-26.
18. Welsh J, McClelland M. Fingerprinting genomes using PCR with arbitrary primers. Nucleic Acids Res. 1990;18(24):7213-8.
19. Williams JGK, Kubelik AR, Livak KJ, Rafalski JA, Tingey SV. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Res. 1990;18(22):6531-5.
20. Jain SK, Neekhra B, Pandey D, Jain K. RAPD marker system in insect study: A review. Indian J Biotechnol. 2010;9(1):7-12.
21. Ballinger-Crabtree ME, Black WC, Miller BR. Use of genetic polymorphisms detected by the random-amplified polymorphic DNA polymerase chain reaction (RAPD-PCR) for differentiation and identification of *Aedes aegypti* subspecies and populations. Am J Trop Med Hyg. 1992;47(6):893-901.
22. Wilkerson RC, Parsons TJ, Albright DG, Klein TA, Braun MJ. Random amplified polymorphic DNA (RAPD) markers readily distinguish cryptic mosquito species (Diptera: Culicidae: *Anopheles*). Insect Mol Biol. 1993;1(4):205-11.
23. Stevens J, Wall R. The use of random amplified polymorphic DNA (RAPD) analysis for studies of genetic variation in populations of the blowfly *Lucilia sericata* (Diptera: Calliphoridae) in southern England. Bull Entomol Res. 1995;85(4):549-55.
24. Çöl B, Tonguç A, Özgül O, Civelek HS, Kaya B. The use of RAPD-PCR analysis in characterization of *Liriomyza trifolii* (Burgess, 1880), *Liriomyza congesta* (Becker 1903), *Agromyza apfelbecki* Strobl, 1902 and *Chromatomyia horticola* (Goureau, 1851) species collected from Turkey. Türk Entomol Derg. 2006;30(4):243-53.
25. Rashit A, Chatterjee RN. Molecular Phylogeny by RAPD Patterns of Drosophilidae (Diptera): RAPD: A Molecular Approach to Assess the Phylogenetic Relationship of the

species of Drosophilidae (Diptera). Saarbrücken, Germany: LAP Lambert Academic Publishing; 2013. 52 p.
26. Malviya S, Tewari RR, Agrawal UR. Genetic Relationship between the Muscids Using RAPD-PCR as marker. Int Res J Biological Sci. 2015;4(1):66-70.
27. Hondelmann P, Borgemeister C, Poehling H-M. Restriction fragment length polymorphisms of different DNA regions as genetic markers in the hoverfly *Episyrphus balteatus* (Diptera: Syrphidae). Bull Entomol Res. 2005;95(4):349-59.
28. Yeh FC, Yang RC, Boyle T. POPGENE Version 1.31. Microsoft Window-based Freeware for Population Genetic Analysis. Edmonton, Canada: University of Alberta; 1999. [accessed: 2016 Mar 13]. Available from: https://sites.ualberta.ca/~fyeh/popgene_download.html.
29. Nei M. Molecular evolutionary genetics. New York: Columbia University Press; 1987. 512 p.
30. Nei M. Analysis of gene diversity in subdivided populations. Proc Natl Acad Sci USA. 1973;70(12):3321-3.
31. Shannon CE, Weaver W. The mathematical theory of communication. Urbana-Champaign: University of Illinois Press; 1949. 144 p.
32. Excoffier L, Laval G, Schneider S. Arlequin (version 3.0): integrated software package for population genetics data analysis. Evol Bioinform Online. 2005;1:47-50.
33. Pavlicek A, Hrda S, Flegr J. Free Tree - Freeware program for construction of phylogenetic trees on the basis of distance data and bootstrap/jackknife analysis of the tree robustness. Application in the RAPD analysis of the genus *Frenkelia*. Folia Biol. 1999;45(3):97-9.
34. Jaccard P. Nouvelles recherches sur la distribution florale. Bull Soc Vaud Sci Nat. 1908;44:223-70.
35. Page RDM. TreeView: An application to display phylogenetic trees on personal computers. Comput Appl Biosci. 1996;12(4):357-8.
36. Peakall R, Smouse PE. GenAlEx 6.5: genetic analysis in Excel. Population genetic software for teaching and research - an update. Bioinformatics. 2012;28(19):2537-9.
37. Nei M, Li WH. Mathematical model for studying genetic variation in terms of restriction endonucleases. Proc Natl Acad Sci USA. 1979;76(10):5269-73.
38. Pritchard JK, Stephens M, Donnelly P. Inference of population structure using multilocus genotype data. Genetics. 2000;155(2):945-59.
39. Falush D, Stephens M, Pritchard JK. Inference of population structure using multilocus genotype data: dominant markers and null alleles. Mol Ecol Notes. 2007;7(4):574-8.
40. Oliveira LO, Venturini BA, Rossi AAB, Hastenreiter SS. Clonal diversity and conservation genetics of the medicinal plant *Carapichea ipecacuanha* (Rubiaceae). Genet Mol Biol. 2010;33(1):86-93.
41. Pinheiro LR, Carregosa Rabbani AR, Da Silva AVC, Da Silva Ledo A, Garcia Pereira KL, Cardamone Diniz LE. Genetic diversity and population structure in the Brazilian *Cattleya labiata* (Orchidaceae) using RAPD and ISSR markers. Plant Syst Evol. 2012;298(10):1815-25.
42. Kopelman NM, Mayzel J, Jakobsson M, Rosenberg NA, Mayrose I. CLUMPAK: a program for identifying clustering modes and packaging population structure inferences across K. Mol Ecol Resour. 2015;15(5):1179-91.
43. Evanno G, Regnaut S, Goudet J. Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. Mol Ecol. 2005;14(8):2611-20.
44. Santos ARF, Ramos-Cabrer AM, Diaz-Hernandez M, Pereira-Lorenzo S. Genetic variability and diversification process in local pear cultivars from northwestern Spain using microsatellites. Tree Genet Genomes. 2011;7(5):1041-56.
45. Rice WR. Analyzing tables of statistical tests. Evolution. 1989;43(1):223-5.
46. Ståhls G, Vujić A, Petanidou T, Cardoso P, Radenković S, Ačanski J, Pérez-Bañón C, Rojo S. Phylogeographic patterns of *Merodon* hoverflies in the Eastern Mediterranean region: revealing connections and barriers. Ecol Evol. 2016;6(7):2226-45.
47. Neetu, Chaudhry S. The Genetic Structure of Anophelines (Culicidae: Diptera): RAPD Versus Allozymes. Proc Zool Soc. 2011;64(1):8-16.