Coordonator:
Dipl.Fiz. Valeria FARBAŞ

Ministerul Educaţiei Naţionale
Ministry of National Education

ACM-V

Asociaţia pentru Cercetare Multidisciplinară
din Zona Vest a României
Association for Multidisciplinary Research
of the West Zone of Romania

Universitatea Politehnica Timişoara
Politehnica University Timişoara

Universitatea de Vest
„Vasile Goldiş" Arad
"Vasile Goldiș" Western
University of Arad

Lucrările celui de al XV-lea Simpozion „TINERII ŞI CERCETAREA MULTIDISCIPLINARĂ"

Proceedings of the XVth Symposium "YOUNG PEOPLE AND MULTIDISCIPLINARY RESEARCH"

14 – 15 Noiembrie 2013
14 – 15 November 2013
Timişoara, România

EDITURA POLITEHNICA
TIMIŞOARA – 2013

Authors: Nevena Veličković, Ljiljana Šašić, Dragana Obreht, Mihajla Djan, Nataša Kočiš Tubić, Ante Vujić

Validation of COI DNA barcoding technique in the taxonomy of genus *Merodon* Meigen (Diptera: Syrphidae)

Key words:
mtDNA, COI, Syrphidae

Address of the authors:
Department of Biology and Ecology
Faculty of Sciences
University of Novi Sad
Trg Dositeja Obradovica 2
21000 Novi Sad, Serbia

Summary

Syrphidae comprises one of the most biologically diverse dipterian families. The genus *Merodon* is widespread in the Mediterranean region and the second largest genus of European hoverflies with more than 50 European species. ***In-depth taxonomic studies*** of *Merodon* in the Europe are very relevant, but the taxonomic status and identification of many Palaearctic *Merodon* species still requires clarification. The aim of this research was to examine mitochondrial gene cytochrome c oxidase I (COI) gene variability in genus *Merodon* and to determine to what extent DNA barcoding can realistically contribute to successful application of molecular taxonomy in the genus. Forty specimens belonging to 4 species groups of the genus *Merodon* sampled over several years from Serbia, Croatia, Montenegro, Macedonia (FYROM) and Greece, ***were*** originally ***identified*** based on ***morphology***. The amplification and sequencing of COI region for all 40 included individuals was successful and the final alignment of the dataset comprised 625 bp. Sequence dataset revealed the presence of 21 haplotypes. Results of phylogenetic analysis, based on Maximum Likelihood consensus tree, showed presence of two to four cryptic species in each of the analyzed species groups.

Introduction

Hoverflies (Diptera: Syrphidae) have been the focus of several studies of taxonomy, biodiversity and conservation in Europe (Rotheray et al. 2001; Ståhls et al., 2003; Speight, 2011). Hoverflies are one of the most important group of pollinators, they interact directly with vegetation because adult hoverflies feed on pollen and nectar. Hoverflies include generalist and cosmopolitan species, which can be very abundant, but also species that are represented by a very low number of individuals because of spatial or temporal restrictions or habitat specialisation (Owen and Gilbert 1989). The genus *Merodon* Meigen is widespread in the Mediterranean region and the second largest genus of European hoverflies with more than 50 European species, excluding Turkey (Speight, 2011).

Since the *Merodon* is species-rich genus numerous studies have dealt with the taxonomy and phylogeography of the genus (Mengual et al., 2006, Ståhls et al.,2009, Vujić et al., 2012; Milankov et al., 2013). Studying phylogenetic relationships within, and genetic distinctiveness of the genus *Merodon* is challenging due to the unclear relationships among taxa and presence of cryptic species. The intra- and interspecific morphological variation found within some groups prompted application of different molecular markers. Results obtained by molecular analyses have partially provided a taxonomic framework for the *M. ruficornis, M. nigritarsis,* and *M. aureus* groups (Vujić et al., 2012), but mtDNA sequence information might not always correspond with species recognised through application of traditional morphological criteria (Mengual et al., 2006; Milankov et al., 2008). Considering certain restrictions of independent application of morphological or molecular characters modern taxonomy developed the principle of the integrative taxonomy covering various aspects of phylogeography, comparative morphology, population genetics, and ecology (Dayart, 2005). Integrated taxonomic studies of *Merodon* in the eastern Mediterranean region of Europe were presented by Vujić et al. (2007, 2012); Ståhls et al. (2009) dealing with the fauna and molecular taxonomy and identification, respectively, of the *Merodon* species from the island of Lesvos (Greece).

The taxonomic status and identification of many Palaearctic *Merodon* species still requires clarification. Using previous experiences, it is clear that each group of species in the genus requires validation of molecular markers in terms of determining their variability, stability, and value as a parameters in integrative taxonomy. The aim of this research was to examine mitochondrial gene cytochrome c oxidase I (COI) gene variability in genus *Merodon* and to determine to what extent DNA barcoding can realistically contribute to successful application of molecular taxonomy in the genus.

Material and Methods

Specimens belonging to 4 species groups of the genus *Merodon* sampled from Serbia, Croatia, Montenegro, Macedonia (FYROM) and Greece, ***were*** originally ***identified*** based on ***morphology*** as *M. rufus*, Meigen 1838 (14 individuals), *M. nanus*, Sack, 1931 (8 individuals), *M. luteomaculatus* Vujić, in litt. (12 individuals), and *M. albifasciatus* Macquart, 1842 (6 individuals).

DNA was extracted from pinned specimens by SDS based method according to Chen et al. (2010). The part of COI mtDNA gene was amplified by C1-J-2183 (alias JERRY) and TL2-N-3014 (alias PAT) primers (Simon et al., 1994) in a 25 µl PCR reaction. The reaction mixture contained 1x PCR buffer, 2.5 mM $MgCl_2$, 0.1 mM of each nucleotide, 1U *Taq* polymerase, 2 pmoles of each primer, and 50 ng template DNA. Amplification was performed in an Eppendorf Personal Thermocycler (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.). Amplification products were checked for the expected size of product by standard 1.5% agarose gel electrophoresis. The remaining product was purified using Exonuclease I and Shrimp

Alkaline Phosphatase enzymes according to the manufacturer's instructions (Fermentas, Lithuania).

Amplicons were sequenced in forward direction and sequences were aligned using the Clustal algorithm implemented in BioEdit 7.0.9.0. (Hall, 1999), with final adjustments by eye. DNA polymorphism (Hd-haplotype diversity, π-nucleotide diversity, k-mean number of pairwise differences) was estimated using DnaSP v5 (Librado and Rozas, 2009). The phylogenetic analysis, the estimation of the best model of nucleotide substitution and nucleotide diversity were calculated in MEGA version 5 (Tamura et al., 2011). The Maximum Likelihood analysis and tree construction were also performed in MEGA vers. 5. Statistical support of internal nodes was calculated with 1000 bootstrap repetitions.

Results and Discussion

Sequencing of mtDNA COI gene was sucessful for all analysed 40 specimens of the genus *Merodon*. The final alignment of the dataset was 625bp, and among those 116 sites were polymorphic, while 113 were parasimony informative. In total 20 different haplotypes belonging to genus *Merodon* were identified. Haplotype diversity value was Hd= 0.951±0.015, while nucleotide diversity value was 0.073. Average number of nucleotide differences was quite high as expected for different genera, 44.01. The individuals were grouped into 4 species according to their morphological classification: *M. rufus, M. albifasciatus, M. nanus* and *M. luteomaculatus*. Haplotype distribution for each species revealed that there are no shared haplotypes among species (Table 1).

Table 1. mtDNA COI haplotype distribution in four species of genus *Merodon*. Absolute number of haplotypes is given for each group, together with relative number per group in parenthesis.

Haplotypes	*M. rufus*	*M. albifasciatus*	*M. nanus*	*M. luteomaculatus*	Total
Hap1			1 (12.5%)		1 (2.5%)
Hap2			4 (50.0%)		4 (10.0%)
Hap3			2 (25.0%)		2 (5.0%)
Hap4			1 (12.4%)		1 (2.5%)
Hap5		3 (50.0%)			3 (7.5%)
Hap6		2 (33.3%)			2 (5.0%)
Hap7		1 (16.7%)			1 (2.5%)
Hap8	1 (7.1%)				1 (2.5%)
Hap9	2 (14.4%)				2 (5.0%)
Hap10	3 (21.4%)				3 (7.5%)
Hap11	1 (7.1%)				1 (2.5%)
Hap12	5 (35.8%)				5 (12.5%)
Hap13	1 (7.1%)				1 (2.5%)
Hap14	1 (7.1%)				1 (2.5%)
Hap15				5 (41.8%)	5 (12.5%)
Hap16				1 (8.3%)	1 (2.5%)
Hap17				3 (25.0%)	3 (7.5%)
Hap18				1 (8.3%)	1 (2.5%)
Hap19				1 (8.3%)	1 (2.5%)
Hap20				1 (8.3%)	1 (2.5%)

Due to faster evolutionary rates compared to mitochondrial rRNA genes, the mitochondrial protein–coding genes are regarded as powerful markers for genetic diversity analysis at lower categorical levels, including families, genera and species (Arif and Khan, 2009). DNA barcoding has become a promising tool for rapid and accurate identification of various taxa and it has been used to reveal unrecognized species in several animal groups, including Syrphidae (Mengual et al., 2006; Ståhls et al., 2009; Vujić et al., 2012; Nedeljković et al., 2013). Furthermore, animal DNA barcodes (600–800 base–pair segments) of the mitochondrial cytochrome oxidase I (COI) gene have been proposed as a tool for quantifying global biodiversity.

In order to test potential of COI sequence variability in the taxonomy of genus *Merodon* (Diptera: Syrphidae) we have performed analysis of the evolutionary history by using the Maximum Likelihood method based on the Tamura 3-parameter model (Tamura, 1992). The rate variation among sites was modeled with a gamma distribution (γ=0.13). The bootstrap test with 1000 replicates was performed (Felsenstein, 1985). All positions containing gaps and missing data were eliminated. The branch positions in the tree had a strong bootstrap support values for all four morphologically determinated species. As expected, COI gene variability proved to be informative molecular marker for distinguishing species from the same genus. Furthermore, results of phylogenetic analysis, based on Maximum Likelihood tree, showed presence of 2 to 4 cryptic species in each of the analyzed species groups (Fig. 1).

In *M. rufus* cluster high bootstrap support value was detected for four separate groups. Detail analysis revealed that one cluster consists of specimens from Olymp mountain (Greece) (RU5 and RU6), second consists of three specimens from Kozuf mountain (FYROM) (RU1, RU2 and RU3) and one specimen from Orjen mountain (Montenegro) has been separated as well. It seems that each high mountain geographically situated in Mediterranaean region provides specific biogeographical conditions which enabled separate speciation processes. Moreover, one group of individuals (RU23, RU24 and RU25) has been clustered together in this *M. rufus* taxon and all originated from mainland Greece. COI barcode provided in depth intraspecific separation for *M. rufus* taxon.

Next, in *M. albifasciatus* cluster two clear groups are separated. Geographically, the clusters correspond to two Greek islands: Rhodos and Crete and high bootstrap values support hypothesis that there actually exist two separate species in *M. albifasciatus* taxon. Similar pattern was found for *M. nanus* species. Specimens originated from Samos island (Greece) clustered together, while separate cluster within consists of specimens from Kopaonik mountain (Serbia), individuals 2,8,N17 and N18 (bootstrap value 97). One sequence, M49, has been separated in *M. luteomaculatus* cluster with high bootstrap support, while other sequences showed moderate level of separation, according to bootstrap values presented. After such position has been obtained, this M49 specimen has been morphologically examined again, and reclassified as *M.* aff. *atratus*. So, grouping within *M. luteomaculatus* species has not been observed such as in other species in this investigation.

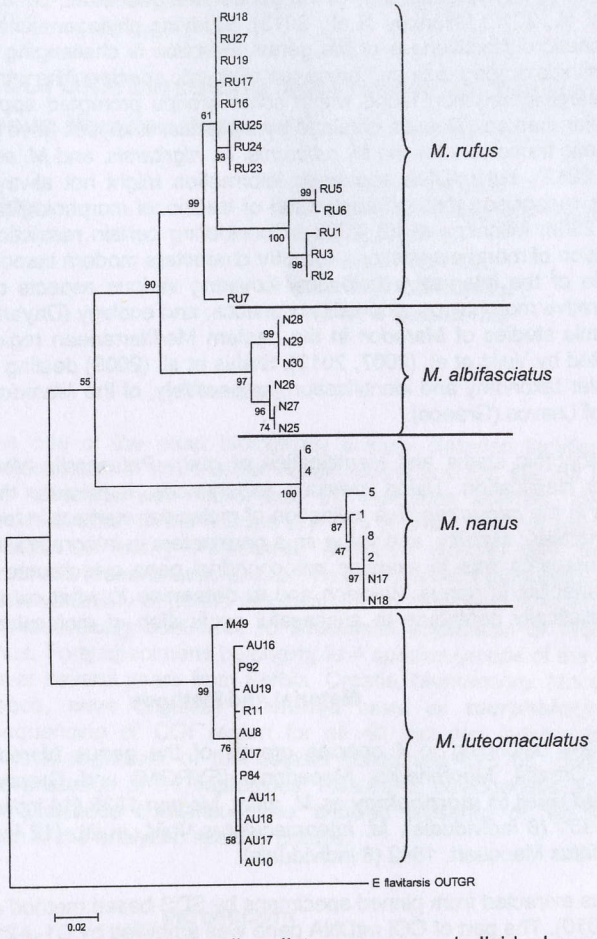

Figure 1. Maximum Likelihood tree revealing distances among individual sequences. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches.

Presented data confirmed usefulness of COI DNA barcoding technique in the taxonomy of genus *Merodon*, and proved the need for integrative taxonomical approach. Moreover, it proved potentiality of selected molecular marker in detection of cryptic taxa. The final validation of these data would require introduction of larger number of specimens from broader geographical region and possibly introduction of additional molecular markers.

Conclusion

Sequencing of mtDNA COI gene was sucessful for all analysed 40 specimens of the genus *Merodon*. In total 20 different haplotypes belonging to genus *Merodon* were identified. Haplotype distribution for each species revealed that there are no shared haplotypes among species and it can be concluded that mtDNA COI fragment is useful and stable marker for determination of interspecies genetic variation. Results of phylogenetic analysis, based on Maximum Likelihood tree, showed presence of two to four cryptic species in each of the analyzed species groups. Presented data confirmed usefulness of COI DNA barcoding technique in the taxonomy of genus *Merodon*, and proved the need for integrative taxonomical approach.

Acknowledgements

This work was financially supported by the Ministry of Education, Science and Tehnological Development of the Republic of Serbia, Grant No. 173002 and the Secretariat for Science and Technological Development, Province of Vojvodina, Grant No. 114-457-2173/2011-01.

References

Arif I. A., Khan H. A. (2009) Molecular markers for biodiversity analysis of wildlife animals: a brief review. Animal Biodiversity and Conservation, 32.1: 9–17.

Chen H, Rangasamy M, Tan SY, Wang H, Siegfried BD (2010) Evaluation of Five Methods for Total DNA Extraction from Western Corn Rootworm Beetles. PLoS ONE 5(8): e11963. doi:10.1371/journal.pone.0011963.

Dayrat B. (2005) Towards integrative taxonomy. Biological Journal of the Linnean Society 85: 407–415.

Felsenstein J. (1985) Confidence limits on phylogenies: An approach using the bootstrap. Evolution 39:783-791.

Hall TA (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for windows 95/98/ NT. Nucleic Acids Symposia Ser 41: 95–98.

Librado P, Rozas J (2009) DnaSP v5: A software for comprehensive analysis of DNA polymorphism data. Bioinformatics 25: 1451-1452.

Mengual X., Ståhls G., Vujić A., Marcos-García M. Á. (2006): Integrative taxonomy of Iberian *Merodon* species (Diptera, Syrphidae). Zootaxa 1377: 1–26.

Milankov V., Ståhls G., Vujić, A. (2008): Molecular diversity of populations of the *Merodon ruficornis* group (Diptera, Syrphidae) on the Balkan Peninsula. Journal of Zoological Systematics and Evolutionary Research 46(2): 143–152.

Milankov V., Ludoški J., Francuski Lj., Ståhls G., Vujić A. (2013) Genetic and phenotypic diversity patterns in *Merodon albifrons* Meigen, 1822 (Diptera: Syrphidae): evidence of intraspecific spatial and temporal structuring. Biological Journal of the Linnean Society 110: 257–280.

Owen J, Gilbert FS (1989) On the abundance of hoverflies (Diptera, Syrphidae). Oikos 55(2):183–193

Rotheray GE, Hancock G, Hewitt S, Horsfield D, MacGowan I, Robertson D, Watt K (2001) The biodiversity and conservation of saproxylic Diptera in Scotland. J Insect Conservat 5:77–85

Simon C., Frati F., Beckenbach A., Crespi B., Liu H., Flook P. (1994) Evolution, weighting and phylogenetic utility of mitochondrial gene sequences and a compilation of conserved polymerase chain reaction primers. Annual Entomological Society of America 87(6): 651-701.

Speight, M.C.D. (2011) Species accounts of European Syrphidae (Diptera), Glasgow 2011. *Syrph the Net, the database of European Syrphidae*, vol. 65, 285 pp., Syrph the Net publications, Dublin.

Ståhls G., Hippa H., Rotheray G., Muona J., Gilbert F. (2003) Phylogeny of Syrphidae (Diptera) inferred from combined analysis of molecular and morphological characters. Systematic Entomology 28: 433–450.

Ståhls G., Vujić A., Peréz-Banoñ C., Radenković S., Rojo S., Petanidou T. (2009) COI barcodes for identification of *Merodon* hoverflies (Diptera, Syrphidae) of Lesvos Island, Greece. Molecular Ecology Resources 9: 1431-1438.

Tamura K. (1992) Estimation of the number of nucleotide substitutions when there are strong transition-transversion and G + C-content biases. Molecular Biology and Evolution 9:678-687.

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S (2011) MEGA5: Molecular Evolutionary Genetics Analysis using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. Mol Biol Evol 28: 2731-2739.

Vujić A., Radenković S., Ståhls G., Ačanski J., Stefanović A., Veselić S., Andrić A., Hayat, R. (2012) Systematics and taxonomy of the *ruficornis* group of genus *Merodon* Meigen (Diptera: Syrphidae). Systematic Entomology 37: 578–602.

Vujić A., Pérez-Bañón C., Radenković S., Ståhls G., Andrić A., Rojo S., Petanidou T., Šimić S. (2007) Two new species of the genus Merodon Meigen 1803 (Diptera: Syrphidae) from the island of Lesvos (Greece), in the eastern Mediterranean. Annales de la Société entomologique de France (N.S.): International Journal of Entomology, 43:(3) 319-326.