DNA barcoding of Croatian faunal biodiversity with notice on caddisflies (Insecta, Trichoptera)

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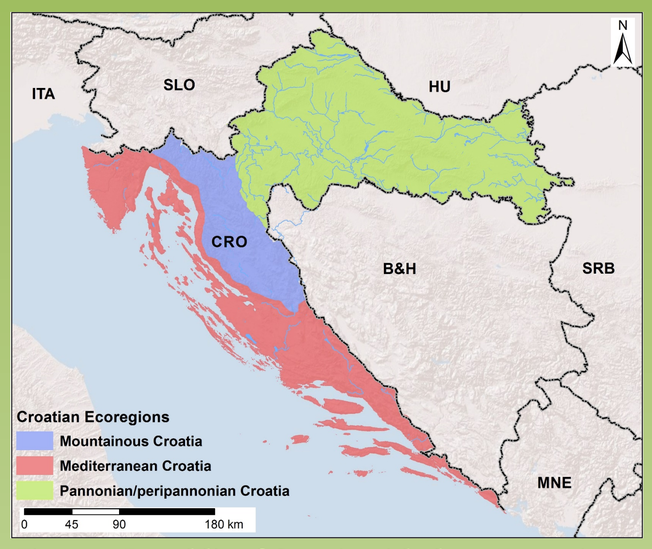

Figure 1. Republic of Croatia with three Ecoregions.

Figure 2. Visovac Lake - National Park Krka.

Introduction

Identification of various animal species is a starting point of research into the morphology, genetics, physiology, distribution, ecology, phylogenetics and other biological characteristics of individual species. Morphological characteristics have for a long time been used as the basis for determination of organisms and this practice is being used until today. Due to a high complexity of diversity of numerous groups of animals, in particular invertebrates, many of which are similar, sibling or cryptic species, other methods are used to support a more exact determination of species and finding those that cannot be determined by conventional morphological methods (Dincă et al. 2013; Previšić et al., 2014).

One of the recent and most frequently used methods in analysis of biodiversity and determination of organisms within a particular area is the DNA barcoding method (Hebert et al. 2003). This method is used to identify species of different groups of organisms (animals, plants, fungi) and is based on sequencing of the standardized segment of the mitochondrial (mt) cytochrome c oxidase subunit 1 (COI) gene (Hebert et al. 2003).
DNA barcoding of caddisflies has overall wide application in association of previously unknown larval stages with adults (e.g., Ruiter et al. 2013), to support description of new species (e.g. Kučinić et al. 2013; Vitecek et al. 2017) and to perform phylogenetic analyses (Vitecek et al. 2017).
Geographically located in the Central and Mediterranean Europe (Fig. 1), Croatian territory has great habitat diversity and represent one of the European biodiversity hotspot. High biodiversity index is direct consequence of very diverse habitats (Figs. 2-6), climatic and hydrological features as well as complex geological history of the region.
The primary goal of the project „DNA barcoding of biodiversity of Croatian fauna" is to investigate the genetic biodiversity and geographic distribution of fifteen groups of Croatian fauna (e.g. Pseudoscorpiones, Ephemeroptera, Plecoptera, Trichoptera) by using DNA barcoding methodology.

Figure 3. The tufa barriers, Plitvice Lake NP.

Results and Discussion

Systematic studies of the biodiversity, distribution, taxonomy and ecological features of caddisflies in the Republic of Croatia have started 20 years ago by researchers at the Croatian Natural History Museum in Zagreb and Faculty of Science, University of Zagreb. In these investigations, 224 Trichoptera species have been recorded so far. Many of these species were found in Croatia for the first time, including descriptions of some new species for science, *Rhyacophila cabrakanensis*, *Chaetopteryx bucari*, *Ecclisoptetyx ivkae* (Malicky et al. 2007; Kučinić et al. 2013; Previšić et al. 2014).
For DNA barcoding in the last four years, about 170 species of Trichoptera have been collected. During this study some new species were identified in Croatian fauna (*Tinodes antonioi*, *Micropterna wageneri*, *Adicella balcanica*, *Adicella cremisa*), and successful DNA barcode analyses were done for 140 caddisflies species (Tab. 1).
DNA barcoding data has shown some very interesting results. In some cases a large genetic diversity among populations of some species from Croatia and other parts of Europe was established. For example Croatian population of *Psychomia pusilla* differs by 10% from populations in Austria and Germany. Mediterranean population of *Triaenodes ochreellus lefkas* in Croatia differs from the population in Spain by 9%. Crenal species *Drusus chrysotus* from mountaions part of Croatia differs from populations in the Alps by 5.4%.
The most interesting results were found for the genus *Diplectrona* for which 5 cryptic species were established in the area of the South East Europe (Fig. 8). These resulats are suppored by both COI andthe nuclear genes analyses.

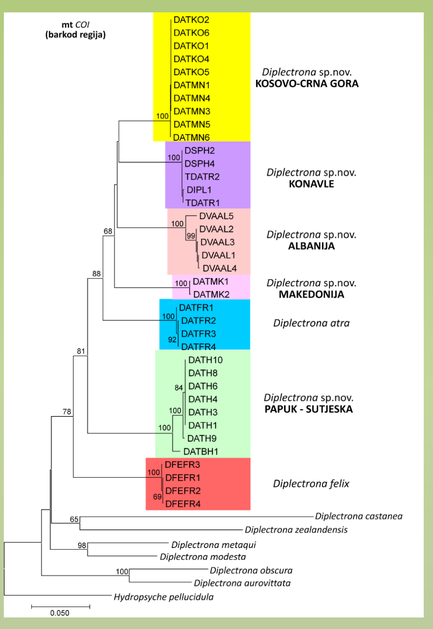

Figure 8. Genus *Diplectrona* – DNA barcoding data, South East Europe.

Material and Methods

Samples of adult caddisflies were collected using entomological net and UV light traps (Fig. 7) in different aquatic habitats in Croatia. The samples were stored in containers with 96% EtOH, for morphological and molecular analysis, respectively. The collected material is deposited in caddisflies' collections in the Croatian Natural History Museum in Zagreb and in the University of Zagreb. Systematic presentaion was done accordinig Malicky (2004) and Morse (2018).
Whole genomic DNA was extracted using GenElute Mammalian Genomic DNA Miniprep kit (Sigma-Aldrich, Germany) according to the manufacturer's specifications and eluted in 100 µl of elution buffer. Full-length COI-5P DNA barcodes were amplified using LCO1490/HCO2198 (Folmer et al. 1994) primer sets. The 50 µl polymerase chain reactions (PCR) mixture contained 1x Go Taq Reaction Buffer (containing 1.5 mM $MgCl_2$, Promega), 0.2 mM of each dNTP, 0.4 µM of each primer, 1.25 units of Go Taq DNA Polymerase (Promega) and 5 µl of DNA eluate. PCR cycling conditions comprised an initial denaturation step (94°C for 2 min) followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 50°C for 30 s and elongation at 72°C for 90 s and a final extension step of 72°C for 7 min. Product purification and bidirectional sequencing was performed by Macrogen Inc. sequencing service (Seoul, South Korea) using the amplification primers. Sequences were edited manually and aligned using the program BioEdit (Hall 1999). DNA sequences were submitted to Barcode of Life Data Systems (BOLD, Ratnasingham & Hebert 2007) and GenBank.

Table 1. Systematic presentation of DNA barcoding caddisflies species from Croatia.

Family Rhyacophilidae
Rhyacophila aurata Brauer, 1857
Rhyacophila balcanica Radovanović, 1953
Rhyacophila cabrakanensis Mal., Prev. & Kuč., 2007
Rhyacophila dorsalis persimilis McLachlan, 1789)
Rhyacophila fasciata Hagen, 1859
Rhyacophila hirticornis McLachlan, 1879
Rhyacophila laevis Pictet, 1834
Rhyacophila loxias Schmid, 1970
Rhyacophila palmeni McLachlan, 1879
Rhyacophila cf. praemorsa McLachlan, 1879
Rhyacophila schmididinarica Urb., Kruš. & Mal., 2000
Rhyacophila torrentium Pictet, 1834
Rhyacophila tristis Pictet, 1834
Rhyacophila vulgaris Pictet, 1834

Family Glossosomatidae
Glossosoma bifidum McLachlan, 1879
Glossosoma conformis Neboiss, 1963
Glossosoma discophorum Klapálek, 1902
Agapetus fuscipes Curtis, 1834
Agapetus laniger Pictet, 1834
Agapetus ochripes Curtis, 1834

Family Hydroptilidae
Hydroptila angulata Mosely, 1922
Hydroptila forcipata Eaton, 1873
Hydroptila vectis Curtis, 1834
Ithytrichia lamellaris Eaton, 1873
Orthotrichia angustella McLachlan, 1865
Orthotrichia tragetti Mosely, 1930
Oxyethira flavicornis Pictet, 1834

Family Philopotamidae
Wormaldia occipitalis (Pictet, 1834)
Wormaldia pulla Mclachlan, 1878
Wormaldia subnigra McLachaln, 1865
Philopotamus montanus (Donovan, 1813)
Philopotamus variegatus (Scopoli, 1763)

FamilyEcnomida
Ecnomus tenellus (Rambur, 1842)

Family Polycentropodidae
Cyrnus trimaculatus (Curtis, 1834)
Neureclipis bimaculata (Linnaeus, 1758)
Plectrocnemia conspersa (Curtis, 1834)
Polycentropus excisus Klapálek, 1894
Polycentropus flavomaculatus (Pictet, 1834)
Polycentropus irroratus (Curtis, 1835)
Polycentropus schmidi Novak & Botosaneanu, 1965

Family Psychomyiidae
Psychomia klapaleki Malicky, 1995
Psychomia pusilla (Fabricius, 1781)
Psychomia sp.
Lype reducta (Hagen, 1868)
Tinodes antonioi Botosaneanu & Tat.-Vigano, 1974
Tinodes braueri McLachlan, 1878
Tinodes dives Pictet, 1834
Tinodes pallidulus McLachlan, 1878
Tinodes unicolor (Pictet, 1834)
Tinodes waeneri (Linnaeus, 1758)

Family Hydropsychidae
Cheumatopsyche lepida (Pictet, 1834)
Diplectrona cf. atra McLachlan, 1878
Diplectrona cf. felix Mclachlan, 1878
Hydropsyche angutipennis (Curtis, 1834)
Hydropsyche bulbifera McLachlan, 1878
Hydropsyche bulgaromanorum Malicky, 1977
Hydropsyche contubernalis McLachlan, 1875
Hydropsyche incognita Pitsch, 1993
Hydropsyche instabilis (Curtis, 1834)
Hydropsyche modesta Navas, 1925
Hydropsyche pellucidula (Curtis, 1834)

Family Phryganeidae
Agrypina varia (Fabricius, 1793)
Phryganea bipunctata Retzius, 1783
Phryganea grandis Linnaeus, 1758
Trichostegia minor Curtis, 1834

Family Brachycentridae
Brachycentrus montanus Klapálek, 1892
Micrasema setiferum (Pictet, 1834)

Family Goeridae
Goera pilosa (Fabricius, 1775)
Silo pallipes (Fabricius, 1781)
Silo piceus Brauer, 1857

Family Lepidostomatidae
Crunoecia kempnyi Morton, 1901
Lasiocephala basalis (Kolenati, 1848)
Lepidosotoma hirtum (Fabricius, 1775)

Family Limnephilidae
Ironoquia dubia (Stephens 1837)
Drusus chrysotus (Rambur, 1842)
Drusus discolor (Rambur, 1842)
Drusus schmidi Botosaneanui, 1953
Drusus vespertinus Marinković-Gospodnetić, 1971
Ecclisopteryx ivkae Previšić, Graf & Vitecek, 2014
Ecclisopteryx keroveci Previšić, Graf & Vitecek, 2014
Grammotaulius nigropunctatus (Retzius, 1783)
Glyphotaelius pellucidus (Retzius, 1783)
Anabolia furcata Brauer, 1857
Rhadicoleptus alpestris Kolenati, 1848
Limnephilus affinis Curtis, 1834
Limnephilus auricula Curtis, 1834
Limnephilus extricatus McLachlan, 1865
Limnephilus flavicornis (Fabricius, 1787)
Limnephilus graecus Schmid, 1965
Limnephilus hirsutus (Pictet, 1834)
Limnephilus ignavus McLachlan, 1865
Limnephilus lunatus Curtis, 1834
Limnephilus marmoratus Curtis, 1834
Limephilus rhombicus (Linnaeus, 1758)
Limnephilus sparsus Curtis, 1834
Limnephilus vittatus (Fabricius, 1798)
Chaetopteryx bosniaca Marinković-Gospod., 1955
Chaetopteryx bucari Kučinić, Szivák & Delić, 2013
Chaetopteryx gonospina Marinković, 1966
Chaetopteryx major McLachalan, 1876
Chaetopteryx marinkovicae Malicky & Krušnik, 1988
Chaetopteryx rugulosa Kolenati, 1848
Chaetopteryx schmidi Botosaneanui, 1957
Annitella apfelbecki Klapálek, 1899
Micropterna lateralis Stephens, 1837
Micropterna nycterobia McLachlan, 1875
Micropterna sequax McLachlan, 1875
Micropterna testacea (Gmelin, 1789)
Micropterna wageneri Malicky, 1971
Stenophylax mitis McLachlan, 1875
Stenophylax permistus McLachlan, 1895
Stenophylax vibex Curtis, 1834
Potamophylax latipennis (Curtis, 1834)
Potamophylax luctuosus Piller & Mitterpacher, 1783
Potamophylax nigricornis (Pictet, 1834)
Potamophylax rotundipennis (Brauer, 1857)
Allogamus auricollis (Pictet, 1834)
Halesus digitatus (Schrank, 1781)
Halesusu tessellatus (Rambur, 1842)
Mesophylax aspersus (Rambur, 1842)

Family Sericostomatidae
Sericostoma flaviorne Schneider, 1845

Family Odontoceridae
Odontocerum albicorne (Scopoli, 1763)

Family Beraeidae
Beraeamyia schmidi Botosaneanu, 1960
Ernodes articularis (Pictet, 1834)

Family Leptoceridae
Adicella balcanica Botosaneanu & Novak, 1965
Adicella cremisa Malicky, 1972
Mystacides longicornis (Linnaeus, 1758)
Mystacides azurea (Linnaeus 1761)
Triaenodes ochreellus lefkas, Malicky 1974
Athripsodes albifrons (Linnaeus, 1758)
Athripsodes aterrimus (Stephens, 1836)
Athripsodes bilineatus (Linnaeus, 1758)
Athripsodes cinereus (Curtis, 1834)
Athripsodes dalmatinus Malicky, 1980
Leptocerus tineiformis Curtis, 1834
Ceraclea dissimilis (Stephens, 1836)
Oecetis furva (Rambur, 1842)
Oecetis lacustris (Pictet, 1834)
Oecetis notata Rambur, 1842

Figure 7. Fieldtrip - UV–lamp.

Figure 4. Spring of the River Cetina.

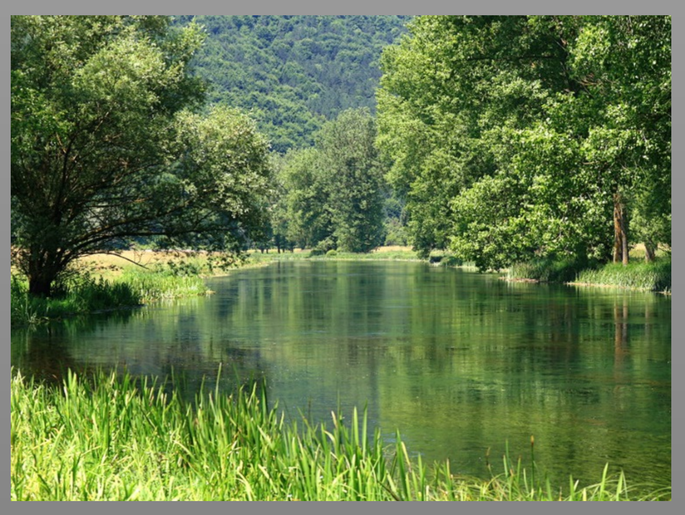

Figure 5. The River Gacka – mountain part of Croatia.

Figure 6. The Prošćansko Lake (Plitvice Lake NP).

Acknowledgments

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