Brazilian Journal of Biology
ISSN 1519-6984 (Print)
ISSN 1678-4375 (Online)

https://doi.org/10.1590/1519-6984.234428
Original Article

DNA barcoding revels first records of three rare coleopteran genera in Northern lakes of Egypt.

D. A. Kheirallah[a]*

[a]Alexandria University, Faculty of Science, Department of Zoology, Alexandria, Egypt
*e-mail: daliakheirallah@yahoo.com

Received: February 26, 2020 – Accepted: May 13, 2020 – Distributed: November 30, 2021
(With 3 figures)

Abstract

One aquatic coleopteran species from family Dytiscidae and two aquatic coleopteran genera from family Hydrophilidae were recorded in the summer period and represent first records in the Egyptian lakes. Beetles were collected from two northern lakes, Lake Idku and Lake Burullus. They were identified by morphological characteristics as well as the mtDNA barcoding method. A molecular phylogenetic approach was used to determine the genetic identity of the collected samples based on the mitochondrial cytochrome oxidase I (COI). *Prodaticus servillianus* (Dytiscidae) from Egypt showed no significant difference in the COI region and they are highly similar to *P. servillianus* from Madagascar. The phylogenetic analysis revealed that the other two coleopteran genera belong to family Hydrophilidae. Based on COI only, there is no clear evidence for their genetic identity at the species level. So, we defined them to the closest taxon and denoted them as *Cymbiodyta* type A and B. The results indicated that resolving the molecular identity of the aquatic beetles from northern lakes of Egypt need more considerations in the field of biological conservation. We concluded that utilization of COI as a barcoding region for identifying some coleopteran species is not sufficient and additional molecular markers are required to uncover the molecular taxonomy at deep levels.

Keywords: Order Coleoptera, DNA Barcoding, mitochondrial cytochrome oxidase I (mtCOI), Northern lakes of Egypt.

O código de barras de DNA revela os primeiros registros de três gêneros raros de coleópteros nos lagos do norte do Egito

Resumo

Uma espécie de coleópteros aquático da família Dytiscidae e dois gêneros de coleópteros aquáticos da família Hydrophilidae foram registrados no período de verão e representam os primeiros registros nos lagos egípcios. Os besouros foram coletados em dois lagos do norte, o lago Idku e o lago Burullus, e identificados por características morfológicas e pelo método de código de barras mtDNA. Uma abordagem filogenética molecular foi usada para determinar a identidade genética das amostras coletadas com base no citocromo oxidase I mitocondrial (COI). Prodaticus servillianus (Dytiscidae) do Egito não mostrou diferença significativa na região COI e é altamente semelhante a P. servillianus de Madagascar. A análise filogenética revelou que os outros dois gêneros de coleópteros pertencem à família Hydrophilidae. Com base apenas no COI, não há evidências claras de sua identidade genética no nível da espécie. Assim, nós os agrupamos no táxon mais próximo e os denominamos Cymbiodyta tipo A e B. Os resultados indicaram que a identidade molecular dos besouros aquáticos dos lagos do norte do Egito precisa de mais considerações no campo da conservação biológica. Concluímos que a utilização de COI como região de código de barras para identificar algumas espécies de coleópteros não é suficiente, sendo necessários marcadores moleculares adicionais para descobrir a taxonomia molecular em níveis profundos.

Palavras-chave: ordem Coleóptera, código de barras de DNA, citocromo oxidase I mitocondrial (mtCOI), Lagos do norte do Egito.

1. Introduction

Few insect species are connected with the marine habitats, most of them belong to the orders Hemiptera, Diptera, and Coleoptera (Agarwala et al., 2013). Among those, Dytiscidae and Hydrophilidae families, belong to order Coleoptera, have many species that occupy brackish estuaries or small ponds (Ghosh et al., 2016). Order Coleoptera is divided into four suborders (Archostemata, Myxophaga, Adephaga, and Polyphaga), from which Adephaga comprises eight truly aquatic families (i.e. Aspidytidae, Gyrinidae, Meruidae, Haliplidae, Noteridae, Hygrobiidae, Amphizoidae, and Dytiscidae), while in Polyphaga, thirteen families habituated marine environment (Helophoridae, Hydraenidae, Epimetopidae, Psephenidae, Cneoglossidae, Hydrochidae, Hydrophilidae, Spercheidae, Elmidae, Scirtidae, Lutrochidae, Dryopidae, and Eulichadidae) (Jäch and Balke, 2008). The adephagan water beetle families were re-examined by Brancucci (1979, 1980, 1981, 1985) and summarized by Wewalka (2004). In the frame of reference of diversity, the most diverse family in suborder Adephaga is Dytiscidae (Nilsson and Holmen, 1995). Around 4000 species inhabit running and still water in all bio-geographical regions (Nilsson, 2001).

Adults and larvae in family Dytiscidae (Suborder: Adephaga) are adapted to aquatic life. They are predacious, feeding on aquatic invertebrates and fish eggs. They can be distinguished from the Hydrophilid beetles by the 1st abdominal sternum and short palpi (Roughley and Larson, 2001). *Hydaticus (Prodaticus) servillianus* (Family: Dytiscidae) is distributed in the whole Afrotropic ecozone, mostly in central Africa and the island of Madagascar (Guignot, 1961). The same species is also known to occur in North America (Roughley and Pengelly, 1981), South America (Trémouilles, 1996), Australia (Watts, 1978), India (Vazirani, 1968), northern Europe (Nilsson, 1981b), and recently in the Arabian Peninsula known from the United Arab Emirates, Oman and Yemen (Hájek and Reiter, 2014).

Polyphagous are found in all types of aquatic habitats except the open ocean (Perkins, 1980; Gerdes et al., 1985; Abellán et al., 2007). Species of aquatic Polyphaga are either scavengers, phytophagous or predatory, particularly in the Hydrophiloidea. They have an essential role in aquatic ecosystems and may significantly affect the trophic structure and functioning of wetland ecosystems. Water beetles of the family Hydrophilidae (Suborder: Polyphaga) are known to be a water scavenger. Genus *Cymbiodyta* has 29 species, entirely Nearctic and rarely Palearctic species (Smetana, 1974; Hansen, 1999). The genus was described by Smetana (1974). Two species have been described, the Old World species of *C. marginella* Fabricius (widespread in Europe, central Asia, and east Kazakhstan) and *C. orientalis* was described by Jia and Short (2011) in southern China. Genus *Cymbiodyta* can be identified by examining the four segmented meso- and metatarsi, unlike the other genera of water scavengers that have 5-5-5 tarsi (except for *Helocombus*) (Jia and Short, 2011). Discovery of the *Cymbiodyta* in the Middle East (Egypt) means that the geographic distribution of the genus was extended.

New recognition methods are required to prevail over the 'taxonomic impediment' (Weeks and Gaston, 1997; Giangrande, 2003). The identification and classification of the biota to the species level constantly count on genetic confirmation (Polyakova et al., 2013). DNA sequence serves as a unique signature and therefore comprise a specific DNA barcode for each species (Polyakova et al., 2013). A phylogenetic analysis and genetic diversity would not only serve to improve the classification of the tribe, but also facilitate the description of the evolution of several character systems such as those associated with sexual conflict (Miller, 2003). Here, we characterize three different genera of aquatic beetles collected from the brackish water of two northern lakes in Egypt. The fauna of water beetles in Egypt stays insufficiently known and the genetic composition of most populations still unclear. This study intended to identify new fauna with first COI-based records in Egypt for such genera.

2. Materials and Methods

The three genera of order Coleoptera were sampled from the northern Egyptian lakes, Lake Idku (Coordinates:31°18′N 30°18′E) and Burullus (Coordinates: 31°29′N 30°52′E) (Figure 1). DNA extracts were prepared from whole body homogenate using the phenol-chloroform extraction method according to (Winnepenninckx et al., 1993). Approximately, 655 bps of the COI gene from mtDNA were amplified using primer combinations HCO and LCO (Folmer et al., 1994). The 25µL PCR reaction mixes included 2.5µL of PCR buffer (10x), 1.25µL of $MgCl_2$ (50mM), 0.5µL of each primer (10µM), 1µL of dNTPs (25mM), 0.25µL of *Taq* polymerase (5U/µL) and 0.5-2.0µL of DNA template (approximately 30ng/µL) to a final volume of 25µL with ultrapure water. For amplification, a Master Eppendorf thermal gradient cycler was used (Brinkmann Instruments, Inc.) with the following setup conditions: 2 min at 95 °C, followed by 35 cycles of 45 sec at 95 °C, 30 sec at 54 °C, and 1 min at 72 °C, and final extension of 10 min at 72 °C. The PCR products were detected on 1.2% agarose gels. The more concentrated products were chosen for the sequencing. Successful amplifications were sequenced at Institute of Biotechnology for Postgraduate Studies and Research, Suez Canal University.

The phylogenetic relationship between COI sequences from the current study and that available from NCBI GenBank were inferred using the Neighbor-joining method (NJ) based on Maximum Composite Likelihood (Tamura et al., 2004). All sequence editing, alignments, and phylogenetic analysis were conducted using MEGA5 (Kumar et al., 2007) with 1000 replications.

3. Results

The COI phylogenetic analysis revealed that the three collected samples are members of Dytiscidae and Hydrophilidae families. The BLAST search on GenBank showed that one sequence is highly similar to COI of *P. servillianus* from Madagascar (% identity= 99.51%, E-value= 0.0, Query cover= 97%). Contrary, the COI of the other two sequences are unique and relatively similar to *C. marginella* (% identity= 87%, E-value= 1e-142, Query cover= 98%). In addition, the NJ phylogenetic tree of *P. servillianus* showed the high supporting bootstrap percentage for *P. servillianus* and *Hydaticus kolbei* with low supporting value at internal nodes of this clade (Figure 2). The other two COI sequences, however, showed non-significant supporting value for their relationship with *C. marginella* under NJ phylogenetic analysis (Figure 3). It is also noticeable that these two sequences were genetically similar in spite of their morphological difference.

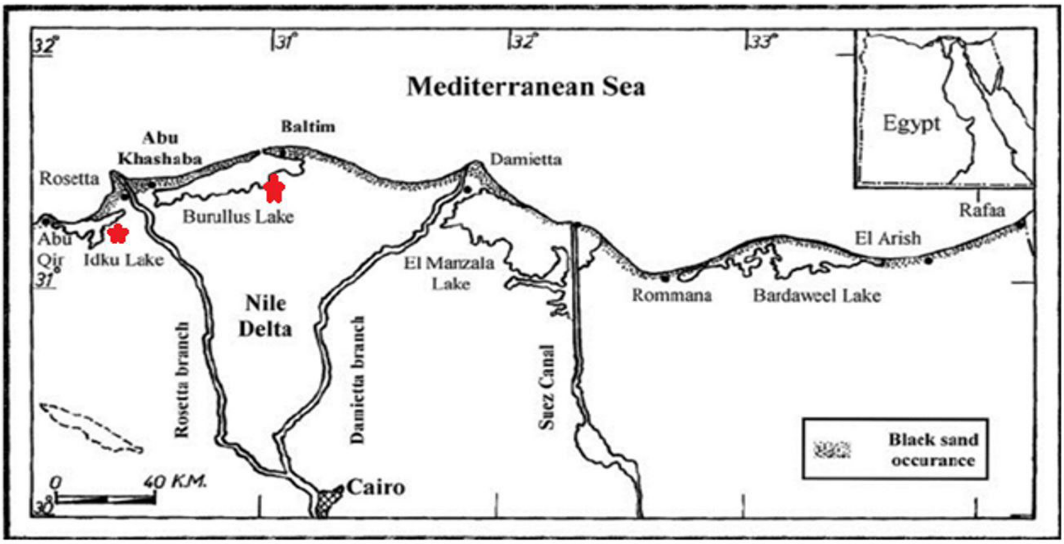

Figure 1. Map of the Egyptian Northern Lakes. Lake Idku and Burullus.

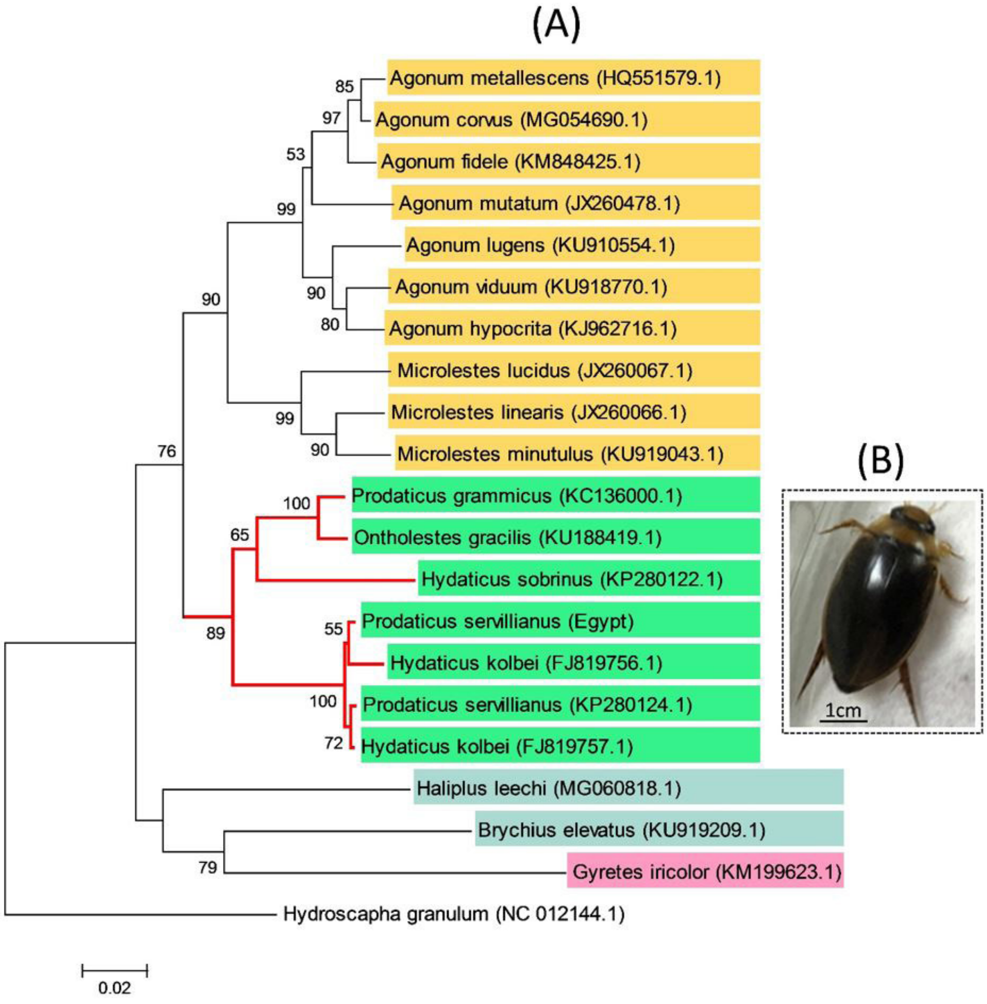

Figure 2. *P. servilianus* collected from Egypt. (A) COI Neighbor-joining tree (NJ) showing the phylogenetic relationship between our sample and that available on GenBank. (B) The morphological features of the species.

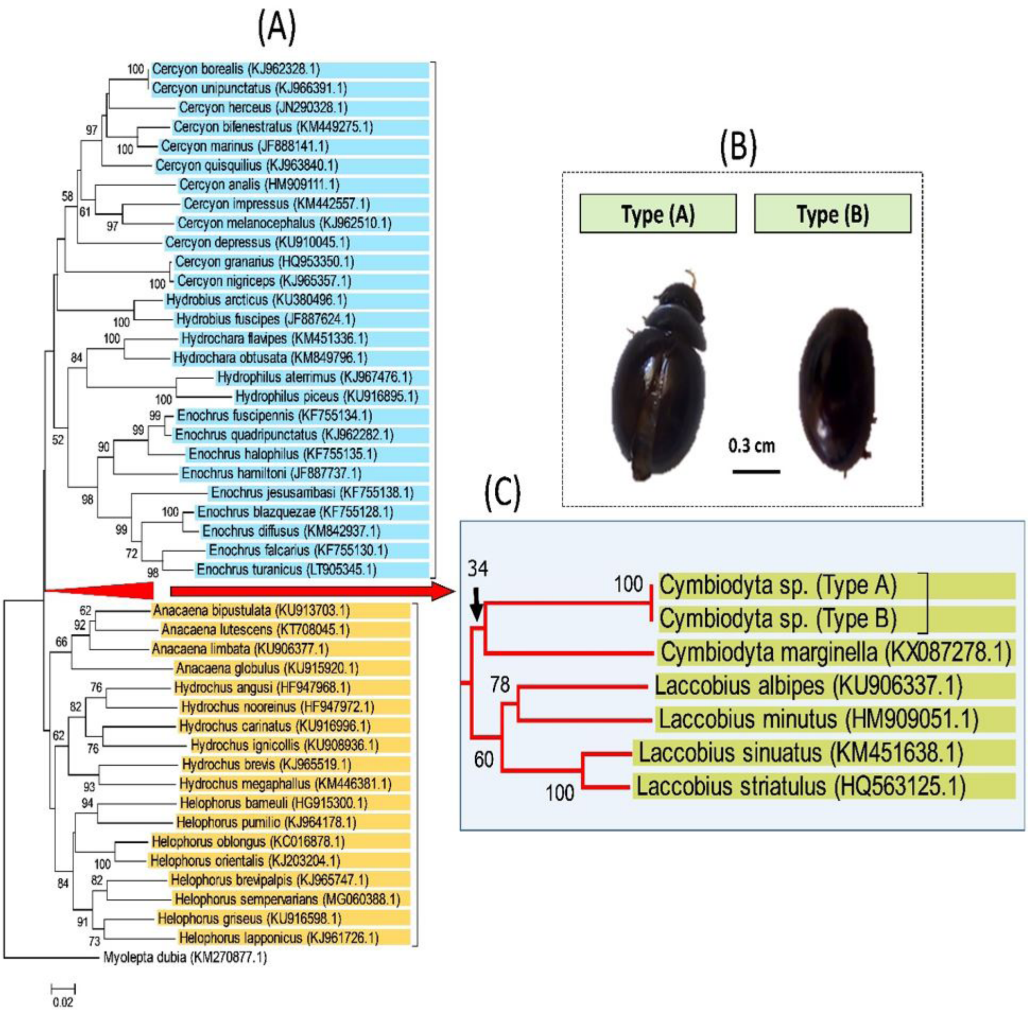

Figure 3. *Cymbiodyta* spp. collected from Egypt. (A) COI Neighbor-joining tree (NJ) showing the phylogenetic relationship between our samples and that available on GenBank. (B) The morphological features of the two species. (C) Detailed phylogenetic relationship between our samples and closely related species on GenBank.

4. Discussion

Insect's diversity reached more than 1 million described species and millions more neither described nor discovered (Grimaldi and Engel, 2005). Insect researches added huge knowledge to understand the bionomics, genetic development, and evolution. Insects are a powerful indicator of water quality (Junqueira et al., 2000) and one of the most relevant indexes to assess them is already being adapted to use molecular tools and metabarcoding (Fernández et al., 2019). Aquatic beetles are an essential part of the biological system, they are powerful indicators of water quality. Yet, additional knowledge about any species can be functional in describing, classifying, and identifying taxa and streams (Ghosh et al., 2016).

The taxonomic inventories of insects considered necessary for the conservations of wetlands. Species identification by conventional morphology is complicated and requires specialist knowledge for describing and naming of new species (Grissell, 1999; Godfray, 2002). It was proposed by Hebert et al. (2003) that a single gene sequence would be satisfied in the discrimination of animal species. They suggested the use of the mitochondrial DNA gene (COI) as an overall bio-identification system for animals. DNA barcoding is succeeded in similar species because of the homogeneity of the DNA sequence (Ashfaq and Hebert, 2016). Recent studies showed that this is generally the case, DNA barcoding identification technique has proven to be effective in discriminating arthropod (Ashfaq and Hebert, 2016) and insect species (Ball and Armstrong, 2006; Liu et al., 2018).

Important advantages of the sequencing identification include the digital nature of a DNA sequence, which allows it to be gathered and interpreted objectively. Furthermore, the extraction of the DNA from any life interval of an organism (egg, larva, and adult) or from the remnants will induce the same identification, whereas traditional identification keys (for instance, holometabolous insects) usually depend on the characteristics of adults (Smith et al., 2005).

So far the three coleopteran genera have been recorded in Egypt: *P. servillianus* and *Cymbiodyta* sp. represent new records for the Egyptian fauna using genetic identification and interspecific distance. The results of the COI barcoding method presented here support the morphological identification and resolve a non-genetically described *Cymbiodyta* species from Egypt. The great potential of the DNA barcode to delimit closely related species is shown in many studies where a single locus was used (mtDNA COI, e.g., Pauls et al., 2010; Jackson et al., 2014). Environmental changes like flood events led to the penetration of numerous brackish and freshwater species like beetles into the lakes from adjacent rivers or wetlands (Perissinotto et al., 2013). This allows a chance to evaluate the diversity and dynamics of beetles within a new aquatic system and combine historical records to provide a baseline for future identification and monitoring of beetle biodiversity patterns in response to environmental changes.

In the present study, although using of CO1 detected the presence of the three coleopteran genera in the two mentioned Egyptian lakes, but it could not separate the two *Cymbiodyta* species which showed a non-significant supporting value for their relationship with *C. marginella* under NJ phylogenetic analysis. CO1 barcoding sequences of specimens are compared to homologous sequences placed in the databases (GenBank, BOLDSystems) for several species identification. Many sequences have been incorrectly explained in the reference libraries so it may generate errors in species identification. Hence, additional molecular markers are needed. A previous morphological studies have described *Cymbiodyta*. Smetana (1974) divided the genus into two groups based on the presence or absence of clear rows of elytral punctures. Those with punctures are distributed only in the western Nearctic region south to Guatemala and Mexico, and the other is found in the western Palearctic region (Hansen, 1999). Jia and Short (2011) reported that *C. orientalis* can be separated from the described genera by other taxonomical characters. The most distinguishable character is the coarsely punctate stria on the elytra. As such, it should be placed in another species group. Hence, by using a gene sequencing it will be easier to identify, group, and separate species. Recording these aquatic beetles in Egypt for the first time represents a great significance in understanding their distribution and abundance.

5. Conclusion

New records of species in Egypt were detected by using DNA barcoding technique which represents a very useful tool in the identification of the species. This technique reveals a new genetic diversity that will affect conservation efforts. It is worth to mention that new genetic species are found in the Egyptian lakes that will create an Egyptian platform for genetic diversity of Egyptian insects.

Acknowledgement

The author are thankful to Molecular Biology Department, faculty of Science, Suez Canal University, Egypt and Zoology Department, faculty of Science, Alexandria University, Egypt.

References

ABELLÁN, P., GÓMEZ-ZURITA, J., MILLÁN, A., SÁNCHEZ-FERNÁNDEZ, D., VELASCO, J., GOLIÁN, J. and RIBERA, I., 2007, Conservation Genetics in Hyper-Saline Inland Waters: Mitochondrial diversity and Phylogeography of an Endangered Iberian Beetle (Coleoptera: Hydraenidae). *Conservation Gen,* vol. 8, pp. 79-88.

AGARWALA, B.K., MAJUMDER, J., DAS, R.K. and MAJUMDER, P., 2013. Aquatic insect fauna and diversity in urban fresh water lakes of Tripura, Northeast India. *Middle East Journal of Scientific Research*, vol. 13, pp. 25-32.

ASHFAQ, M. and HEBERT, P.D., 2016. DNA Barcodes for bio-surveillance: regulated and economically important arthropod plant pests. *Genome*, vol. 59, no. 11, pp. 933-945. http://dx.doi.org/10.1139/gen-2016-0024. PMid:27753511.

BALL, S.L. and ARMSTRONG, K.F., 2006. DNA Barcodes for insect pest identification: A test case with Tussock Moths (Lepidoptera: Lymantriidae). *Canadian Journal of Forest Research*, vol. 36, no. 2, pp. 337-350. http://dx.doi.org/10.1139/x05-276.

BRANCUCCI, M., 1979. Insects of Saudi Arabia. Coleoptera: Fam Haliplidae, Dytiscidae, Gyrinidae. *Fauna Saudi Arabia*, vol. 1, pp. 156-161.

BRANCUCCI, M., 1980. Insects of Saudi Arabia. Coleoptera: Fam. Haliplidae, Dytiscidae, Gyrinidae. Part 2. *Fauna Saudi Arabia*, vol. 2, pp. 102-111.

BRANCUCCI, M., 1981. Insects of Saudi Arabia. Coleoptera: Fam. Dytiscidae (Part 3). *Fauna Saudi Arabia*, vol. 3, pp. 227-230.

BRANCUCCI, M., 1985. Insects of Saudi Arabia. Coleoptera: Fam. Haliplidae, Noteridae, Dytiscidae, Gyrinidae (Part 4). *Fauna Saudi Arabia*, vol. 6, pp. 229-242.

FERNÁNDEZ, S., RODRIGUEZ-MARTINEZ, S., MARTINEZ, J.L., GARCIA-VAZQUEZ, E. and ARDURA, A., 2019. How can eDNA contribute in riverine macroinvertebrate assessment? A metabarcoding approach in the Nalón River (Asturias, Northern Spain). *Environmental DNA.*, vol. 1, no. 4, pp. 385-401. http://dx.doi.org/10.1002/edn3.40.

FOLMER, O., BLACK, M., HOEH, W., LUTZ, R. and VRIJENHOEK, R., 1994. DNA primers for amplification of mitochondrial Cytochrome C Oxidase Subunit I from diverse metazoan invertebrates. *Molecular Marine Biology and Biotechnology*, vol. 3, no. 5, pp. 294-299. PMid:7881515.

GERDES, G., SPIRA, J. and DIMENTMAN, C., 1985. The Fauna of the Gavish Sabkha and the Solar Lake – A Comparative Study. In: G. FRIEDMAN and W. KRUMBEIN, eds. *Ecological Studies. Hypersaline Ecosystems*. Berlin: Springer Verlag, 486 p.

GHOSH, S., DAS, P. and MITRA, B., 2016. Studies on the fresh water aquatic beetle fauna (Coleoptera: Insecta) of Chilika Lake and its adjoining areas and their colonization in brackish water. *International Journal of Fauna Biological Studies*, vol. 3, pp. 19-24.

GIANGRANDE, A., 2003. Biodiversity, conservation, and the 'Taxonomic Impediment'. *Aquatic Conservation*, vol. 13, no. 5, pp. 451-459. http://dx.doi.org/10.1002/aqc.584.

GODFRAY, H.C., 2002. Challenges for taxonomy. *Nature*, vol. 417, no. 6884, pp. 17-19. http://dx.doi.org/10.1038/417017a. PMid:11986643.

GRIMALDI, D.A. and ENGEL, M.S., 2005. *Evolution of the Insects*. Cambridge: Cambridge University Press, 733 p.

GRISSELL, E.E., 1999. Hymenopteran Biodiversity: Some Alien Notions. *American Entomologist*, vol. 45, no. 4, pp. 235-244. http://dx.doi.org/10.1093/ae/45.4.235.

GUIGNOT, F., 1961. Revision of African Hydrocanthares (Coleoptera: Dytiscoidea), 3rd Part. Annals of the Royal Belgian Congo Museum, Series 8vo. *Zoological Science*, vol. 90, pp. 659-995.

HÁJEK, J. and REITER, A., 2014. Adephagous water beetles (Coleoptera: Gyrinidae, Haliplidae, Noteridae, Dytiscidae) of Yemen and Dhofar Region (Oman) with description of a new *Hyphydrus* from Socotra Island. *Acta Entomologica Musei Nationalis Pragae*, vol. 54, pp. 63-99.

HANSEN, M., 1999. *World Cataloque of Insects - Hydrophiloidea (Coleoptera)*. Denmark: Apollo Books, Stenstrup, 2416 p.

HEBERT, P.D., CYWINSKA, A., BALL, S.L. and DEWAARD, J.R., 2003. Biological identifications through DNA barcodes. *Proceedings. Biological Sciences*, vol. 270, no. 1512, pp. 313-321. http://dx.doi.org/10.1098/rspb.2002.2218. PMid:12614582.

JÄCH, M.A. and BALKE, M., 2008. Global diversity of water beetles (Coleoptera) in Freshwater. *Hydrobiologia*, vol. 595, no. 1, pp. 419-442. http://dx.doi.org/10.1007/s10750-007-9117-y.

JACKSON, J.K., BATTLE, J.M., WHITE, B.P., PILGRIM, E.M., STEIN, E.D., MILLER, P.E. and SWEENEY, B.W., 2014. Cryptic biodiversity in streams: a comparison of macroinvertebrate communities based on morphological and DNA Barcode Identifications. *Freshwater Science*, vol. 33, no. 1, pp. 312-324. http://dx.doi.org/10.1086/675225.

JIA, F. and SHORT, A.E., 2011. Description of *Cymbiodyta orientalis* sp. n., the First species of the Genus from the oriental region (Coleoptera: hydrophilidae). *Entomological News*, vol. 121, no. 4, pp. 348-351. http://dx.doi.org/10.3157/021.121.0408.

JUNQUEIRA, M.V., AMARANTE, M.C., DIAS, C.F.S. and FRANÇA, E.S., 2000. Biomonitoramento da qualidade das águas da Bacia do Alto Rio das Velhas (MG/Brasil) através de macroinvertebrados. *Acta Limnologica Brasiliensia*, vol. 12, pp. 73-87.

KUMAR, N.P., RAJAVEL, A.R., NATARAJAN, R. and JAMBULINGAM, P., 2007. DNA Barcodes can distinguish species of Indian Mosquitoes (Diptera: culicidae). *Journal of Medical Entomology*, vol. 44, no. 1, pp. 1-7. http://dx.doi.org/10.1093/jmedent/41.5.01. PMid:17294914.

LIU, L., GUO, Z., ZHONG, C. and SHI, S., 2018. DNA Barcoding reveals insect diversity in the mangrove ecosystems of Hainan Island, China. *Genome*, vol. 61, no. 11, pp. 797-806. http://dx.doi.org/10.1139/gen-2018-0062. PMid:30398899.

MILLER, K.B., 2003. The phylogeny of diving beetles (Coleoptera: Dytiscidae) and the evolution of sexual conflict. *Biol. J. Linnaean Soc.*, vol. 79, no. 3, pp. 359-388. http://dx.doi.org/10.1046/j.1095-8312.2003.00195.x.

NILSSON, A.N. and HOLMEN, M., 1995, *The Aquatic Adephaga (Coleoptera) of Fennoscandia and Denmark. II. Dytiscidae*. Leiden: E J Brill, 192 p.

NILSSON, A.N., 1981b. The Fennoscandian species of the genus *Hydaticus* Leach (Coleoptera: dytiscidae). *Entomologica Scandinavica*, vol. 12, no. 1, pp. 103-108. http://dx.doi.org/10.1163/187631281X00427.

NILSSON, A.N., 2001. Dytiscidae (Coleoptera). *World Catalogue Insects*, vol. 3, pp. 1-395.

PAULS, S.U., BLAHNIK, R.J., ZHOU, X., WARDWELL, C.T. and HOLZENTHAL, R.W., 2010. DNA barcode data confirm new species and reveal cryptic diversity in Chilean *Smicridea* (Smicridea) (Trichoptera: hydropsychidae). *Journal of the North American Benthological Society*, vol. 29, no. 3, pp. 1058-1074. http://dx.doi.org/10.1899/09-108.1.

PERISSINOTTO, R., STRETCH, D.D. and TAYLOR, R.H., 2013. *Ecology and conservation of estuarine ecosystems: Lake St. Lucia as a Global Model.* England: Cambridge University Press, 486 p. http://dx.doi.org/10.1017/CBO9781139095723.

PERKINS, P.D., 1980. Aquatic beetles of the Family Hydraenidae in the western Hemisphere: classification, biogeography and inferred Phylogeny (Insecta: Coleoptera). *Quaestiones Entomologicae*, vol. 16, pp. 3-554.

POLYAKOVA, N., BOUTIN, A. and BRYKOV, V., 2013. Barcoding and phylogenetic inferences in Nine Mugilid Species (Pisces, Mugiliformes). *Animal Systematics Evolution Diversity*, vol. 29, no. 4, pp. 272-278. http://dx.doi.org/10.5635/ASED.2013.29.4.272.

ROUGHLEY, R.E. and LARSON, D.J., 2001. Dytscidae Leach, 1815. In: R.H. ARNETT and M.C. THOMAS, eds. *American beetles. Archostemata, Myxophaga, Adephaga, Polyphaga: Staphyliniformia*. New York: CRC Press, pp. 156-186.

ROUGHLEY, R.E. and PENGELLY, D.H., 1981. Classification, phylogeny, and zoogeography of *Hydaticus* Leach (Coleoptera: Dytiscidae) of North America. *Quaestiones Entomologicae*, vol. 17, pp. 249-314.

SMETANA, A., 1974. Revision of the Genus *Cymbiodyta* Bed. (Coleoptera: hydrophilidae). *Memoirs of the Entomological Society of Canada*, vol. 93, no. S93, pp. 1-113. http://dx.doi.org/10.4039/entm10693fv.

SMITH, M.A., FISHER, B.L. and HEBERT, P.D., 2005. DNA barcoding for effective biodiversity assessment of a hyper diverse arthropod group: The ants of Madagascar. *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences*, vol. 360, pp. 1825-1834. http://dx.doi.org/10.1098/rstb.2005.1714.

TAMURA, K., NEI, M. and KUMAR, S., 2004. Prospects for inferring very large phylogenies by using the neighbor-joining method. *Proceedings of the National Academy of Sciences of the United States of America*, vol. 101, no. 30, pp. 11030-11035. http://dx.doi.org/10.1073/pnas.0404206101. PMid:15258291.

TRÉMOUILLES, E.R., 1996. Review of the *Hydaticus* leach genus in South America, with description of three new species (Coleoptera, Dytiscidae). *Physis Sections B*, vol. 52, pp. 15-32.

VAZIRANI, T.G., 1968. Contribution to the study of aquatic beetles (Coleoptera). 1. on a collection of Dytiscidae from the western ghats with descriptions of two new species. *Oriental Insects*, vol. 1, no. 1-2, pp. 99-112. http://dx.doi.org/10.1080/00305316.1967.10433855.

WATTS, C.H., 1978. A Revision of the Australian Dytiscidae (Coleoptera). *Australian Journal of Zoology Supplementary Series*, vol. 26, no. 57, pp. 1-166. http://dx.doi.org/10.1071/AJZS057.

WEEKS, P.J. and GASTON, K.J., 1997. Image analysis, neural networks, and the taxonomic impediment to biodiversity studies. *Biodiversity and Conservation*, vol. 6, no. 2, pp. 263-274. http://dx.doi.org/10.1023/A:1018348204573.

WEWALKA, G., 2004. Dytiscidae (Insecta: Coleoptera) of the Socotra archipelago, with descriptions of two new species. *Fauna Arabia*, vol. 20, pp. 463-472.

WINNEPENNINCKX, B., BACKELJAU, T. and DE WACHTER, R., 1993. Extraction of high molecular weight DNA from Molluscs. *Trends in Genetics*, vol. 9, no. 12, pp. 407. http://dx.doi.org/10.1016/0168-9525(93)90102-N. PMid:8122306.