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Mitochondrial gene marker sequences reveal identities of gobies at Minalungao National Park, Nueva Ecija, Philippines

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Abstract

Gobiidae is a rapidly growing family besides being the largest, with new species continuously discovered in all parts of the world, specifically in Asia and in the Philippines. Given this diversity, this study provides an initial identification and is the first report of gobies found at Minalungao National Park, a Key Biodiversity Area (KBA) in Central Luzon, Philippines. Goby species were identified molecularly using mitochondrial genes namely: CO1 (Cytochrome Oxidase 1), Control Region or D-Loop, and 16s rRNA. Samples were collected along Minalungao River. DNA was extracted from muscle tissues and gene markers were amplified using polymerase chain reaction. Sequences were subjected to BLAST for species identification. Results showed CO1 sequences ranging from 712-719 bp; D-Loop at 781-841 bp; and 559-592 bp in 16srRNA. BLAST sequences revealed four species of gobies belonging to 2 genera namely *Rhinogobius giurinus* (99% maximum identity using 16srRNA, 95% with D-Loop), *Rhinogobius brunneus* (97% matched using CO1), *Glossogobius bicirrhosus* (82% matched with D-Loop), and *Glossogobius giuris* (with 87% CO1 match).

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Introduction

The Philippines is one of the mega diverse countries that holds around 80% of the world's biodiversity (Conservation International, 2006; DENR-PAWB, 2006) with the highest form of endemism in an area. While most fish biodiversity studies are focused on marine species, researches on inland or freshwater fishes are mostly focused on commercially important species. With more endemic species to be discovered, biodiversity and identification studies are warranted. Among these fishes are the gobies under the Family Gobiidae. It is largely marine though with fresh water representatives, with newer species continuously discovered and named, mostly in Asia and in the Philippines where approximately 146 species of Gobiidae are known to occur with seven (7) endemic species and around 68 species native to the Philippines.

Among the areas that needs to be assessed for gobies is the Minalungao National Park, a Key Biodiversity Area (KBA) in Central Luzon, Philippines. Through the efforts of the government to protect and conserve its natural resources, Minalungao forests was designated as a protected National Park. It covers 2,018 hectares of thick, unexplored forests and affluent rivers, and indeed, requires attention for conservation. Thus, a biodiversity assessment is imperative to help design better plans for its protection and conservation.

Accordingly, biodiversity assessment requires species identification. To accurately identify the species, molecular markers are employed. Mitochondrial markers are extensively used in identification and phylogenetic studies in fishes because of its rapid rate of evolution (Chauhan and Rajiv, 2010). Most molecular identification studies in fishes have relied on the analysis of mitochondrial DNA (mtDNA) sequence variation because of its maternal inheritance and high rate of mutation (Liu and Cordes, 2004; Angers and Bernatchez, 1998; Sato *et al.*, 2003). Among these markers are the CO1 (Cytochrome oxidase 1), used as a standard barcode marker in fishes (Ward *et al.*, 2005); the Control Region or D-Loop, for studying genetic variation among closely related individuals because of its highly divergent sequences (Walberg and Clayton, 1981), high mutation accumulation (Xie *et al.*, 2006) and fastest mutation rate among the mitochondrial genes; and the 16s rRNA, a common molecular marker to identify commercial fish and seafood species (Rasmussen and Morrisey, 2008) routinely used for determining phylogeny in fishes. This study provides an initial inventory of Gobiidae at Minalungao National Park, Philippines.

Materials and methods

Sample collection

The fish samples were collected from three sites along Minalungao River (15°17'52.36" N). Samples chosen for molecular identification were based on phenotypes and overall appearance. Goby fishes were quickly identified based on the description of Herre (1927) by having a fused ventral fin, unstalked eyes, thick lips and laterally compressed belly. Muscle tissue samples were preserved in 95% ethanol and stored at -20°C prior to DNA extraction. Voucher specimens were preserved in formaldehyde.

DNA extraction

Genomic DNA from muscle tissue was extracted using DNeasy Tissue Kit (Qiagen® Group, Hilden Germany) following the manufacturer's protocol. About 180 µl Buffer ATL and 20µl proteinase K were mixed to homogenized tissue before incubation at 56°C. About 200 µl buffer AL and 200 µl of 95% ethanol were then added and vortexed, and the samples pipetted to the DNeasy Mini Spin Column to centrifuge at 8000 rpm for 1 min. The supernatant was discarded and the DNeasy Mini Spin Column was transferred to a new 2 ml tube. Five hundred microliter (500 µl) Buffer AW1 was dispensed to the sample and was centrifuged for 3 min. The flow-through was discarded again and the DNeasy Mini Spin Column was placed in a new 1.5 ml tube. Buffer AE was directly pipetted to the DNeasy Mini Spin Column membrane before subjecting to final centrifugation.

Polymerase chain reaction

The target regions of the mitochondria were amplified using the specific primer pairs: COI-F (GGTCAACAAATCATAAAGATATTGG) and COI-R (TAAACTTCAGGGTGACCAAAAAATCA); 16SH (5'-CCGGTCTGAACTCAGATCACGT-3') and 16SL (5'-GTTTACCAAAAACATGGCTTC-3') (Heralde III *et al*., 2010); Ormt-449UP (5'-CTAACTCCCAAAGCTAGGATTCT-3') and Oreo12S-L (5'-CCCAGTTTGTTTCGCAGCTTTCGT-3') (Samonte *et al*., 2003). PCR amplification was performed using a final volume of 30 µl comprised of 19.1 µl sterile distilled water, 3.0 µl PCR Buffer, 3.0 µl MgCl2, 1.2 µl dNTP's, 1.2 µl forward primer, 1.2 µl reverse primer, 0.3 µl Taq Polymerase Recombinant and 1.0 µl DNA template. PCR reaction was performed in a 96 – well programmable thermal cycler (Veriti) with the following program: initial denaturation of 94°C for 5 minutes; 35 cycles of denaturation for 1 minute at 94°C; annealing for 90 secs at 50°C; extension for 90 secs at 72°C; and a final extension of 5 min at 72°C with a hold of 4°C.

DNA sequencing

The PCR products were sent to the Philippine Genome Center at National Institute of Molecular Biology and Biotechnology in University of the Philippines-Diliman, Quezon City, Philippines for purification and sequencing. Sequences were assembled using the Chromaslite and were subjected to Basic Local Alignment Search Tool (BLAST, NCBI) for identification.

Results and discussion

The range of the contiguous sequences generated for CO1 were at 712bp and 719 bp; the Control region (D-loop) at 781-841 bp; and 16S rRNA from 558 to 608 bp). Using NCBI BLAST, CO1 sequences revealed the identities of *Rhinogobius brunneus* (97%) and *Glossogobius giuris*(87%). 16s and D-Loop (Control Region) revealed *Rhinogobius giurinus* (99%)and *Glossogobius bicirrhosus* species (82%).

The identity match of the sequences to *Rhinogobius brunneus* using two gene markers were congruent to the occurrence and diversity of this species in the Philippines and in other Asian countries where it is widely distributed (Chen *et al.*, 1999; Suk &Choe 2002; Froese & Pauly, 2006) where adults are generally found in lakes and rivers. This identification could help contribute to a baseline data for its conservation as this species faces a number of potential threats such as pollution and hydrological projects like dams and water diversion projects(Devi &Boguskaya, 2009) as well as overfishing for food consumption.Given a wider sampling area and collection time, it is supposed that more variants can be characterized from this species as they are generally known with several phenotypes based on shape and coloration which tend to isolated according habitat(fishbase.org), of which molecular identification is particularly useful. This supposition is true with the identification of another *Rhinogobius* species, *R. giurinus*through two gene markers, 16s and D-Loop, which are more sensitive markers to delineate closely related species. This identification comes as no surprise considering that this species have a large distribution area and has no known major widespread threats and has been assessed with a status of Least Concern in the IUCN list (Huckstorf, 2012). It has a wide distribution in Asia, as well and originally distributed over China, Taiwan, Korean, Japan, Bonin Islands, Ryukyu Islands and from Red river basin and Viet Nam (Masuda *et al*., 1984; Chen & Shao, 1996, Xie*et al*., 2001; Ngyuen, 2005, Serov*et al*., 2006). This identification may serve as initial data on this species, especially those inhabiting Minalungao National Park as there is no concrete information available on the species population yet but was reported to have been caught in small trap nets in rivers with less abundance (Masuda *et al*., 1984). They are found from large rivers to streams and in shallow waters over sandy bottom (Serov *et al*., 2006). They are also found both upstream and estuaries, are carnivorous and lay their eggs under stones in river shoals and the larvae travel in both river and the sea and return to rivers (Masuda *et al*. 1984).

All the three gene markers used provided the identity for the *Glossogobius*species. Two *Glossogobius* species were identified, *G. giuris and G. bicirrhosus*. For*G. bicirrhosus*no species identity matched with the sequences using 16sRNA. However, D-Loop provided species identification at 82%. Similar with the *Rhinogobius* species, its identificationis consistent with its reported large distribution area with no known major widespread threats to the species (Larson, 2012), which are found mainly in streams or sand bottoms (Allen, 1991). It is predominantly distributed in Indonesia, Philippines, Taiwan and Japan and is also reported from Melanesia, north-eastern Australia and Papua New Guinea. (Talwar & Jhingran, 1991). This species has a status of Least Concern at the IUCN Red List (2018). Comparable with the collected samples, *G. giuris* is usually characterized phenotypically with a brownish-yellow body as well as rounded, dark spots on the sides. Other specimens display mimicked coloration of the substrates, hence, dark and ivory coloration are observed (Keith *et al.*, 1999).

This study confirms previous reports that diversity of gobies are mostly found along this part of Luzon (Herre, 1927). Herre (1927) particularly mentioned that it is only along the North of the Philippines that they occur in such enormous quantities that their capture and preservation form one of the chief resources of the region and one of the most valuable sources of income. Endemism of gobies to inland waters are due to the archipelagic nature of the Philippines that in turn, contains various flora and fauna in its landscapes and seascapes (Vedra *et al.*, 2013). Studies on this should be a priority for the reason that high level of endemism of these fishes is a significant indicator of the local biodiversity (Sanda & Kovacic, 2009; Bagorodsky *et al.*, 2010).

Few studies were conducted despite goby species diversity (UPLB Limnological Research Station, 2011) in the Philippines. These studies usually utilize morphological characters and morphometrics in identifying goby fishes (Akihito & Meguro, 1975; Kok & Blaber, 1976; Miller & Wongrat, 1979; Parenti & Maciolek, 1993; Chen *et al.*, 1995; Chen *et al.*, 1997; Chen *et al.*, 1999; Chen & Kottelat, 2003; Chen & Tan, 2005; Chen & Fang, 2006; Huang & Chen, 2007; Shibukawa & Aonuma, 2007; Jaafar & Larson, 2008; Hossain *et al.*, 2009; Greenfield & Jewett, 2011; Myers *et al.*, 2012; Froese & Pauly 2012; Kannan *et al.*, 2013). This leaves molecular identification of goby fishes bare and sparse especially in the Philippines (Keith *et al.*, 2010). This molecular identification of gobies at Minalungao National Park serves as baseline information for the gobies present in the area which could help in their conservation and aid in the prevention of potential threats to these species.

Conclusion

The mitochondrial markers were successful in the identification of gobies at Minalungao National Park. Four species belonging to two genera were identified: *Rhinogobius brunneus, Rhinogobius giurinus, Glossogobius giuris* and *Glossogobius bicirrhosus* species. While only 4 species were identified in this study, we hypothesize that a large number of gobies can still be discovered and named with improved collection, timing, and more robust technology, hence, identification of more species is recommended.

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References

Akihito Y, Meguro K. 1975. First record of Glossobius celebius from Japan. Japanese Journal of Icthyology**21(4),** 227-230.

Allen GR. 1991. Field guide to the freshwater fishes of New Guinea. Publication, no. 9. 268 p. Christensen Research Institute, Madang, Papua New Guinea.

Angers B, Bernatchez L. 1998. Combined Use of SMM and Non-SMM Methods to Infer Fine Structure and Evolutionary History of Closely Related Brook Charr (Salvelinus fontinalis, Salmonidae) Populations from Microsatellites.Molecular Biology and Evolution**15(2),**143–159. 1998

Bagorodsky S, Kovacic M, Ozen O, Bilecenogin M. 2010. Records of two uncommon goby species (Millerigobius macrocephalus, Zebrus zebrus) from the Aegean Sea. Acta Adriatica **51(2),**217- 222.

Bucol AA, Carumbana EE. 2010. Checklist of fishes found in the fresh and brackish waters of Negros and Siquijor, Philippines. Asian Scientific Journals **1,**1.

Carpenter KE, Springer VG. 2005. Environmental Biology of Fishes **72,** 467-480.

Chauhan T,Raijiv K. 2010. Molecular markers and their applications in fisheries and aquaculture. Advances in Bioscience and Biotechnology, 2010 **1,** 281-291.

Chen IS, Han CC, Fang LS. 1995. A new record of freshwater gobiid fish, Schismatogobius roxasi (Pisces, Gobiidae) from South-eastern Taiwan. Bulletin of the National Museum of Nature and Science**6,**135-137.

Chen IS, Shao KT. 1996. A taxonomic review of the gobiid fish genus Rhinogobius Gill, 1859, from Taiwan, with descriptions of three new species. Zoological Studies **35(3),**200-214.

Chen IS, Hsu CH, Hui CF, Shao KT, Miller PJ, Fang LS. 1997. Sequence length and variation in the mitochondrial control region of two freshwater gobiid fishes belonging to Rhinogobius (Teleostei: Gobioidei).Journal of Fish Biology **53,** 179–191.

Chen IS, Wu HL, Shao KT. 1999. A new species of Rhinogobius (Teleostei: Gobiidae) from Fujian Province, China. Ichthyological Research **46(2),**171 - 178.

Chen I, Kottelat M, Miller PJ. 1999. Freshwater gobies of the genus Rhinogobius from the Mekong basin in Thailand and Laos, with descriptions of three new species. Zoological Studies **38,** 19-32.

Chen IS, Kottelat M. 2003. Three new freshwater gobies of the genus Rhinogobius (Teleostei: Gobiidae) from North-eastern Laos. The Raffles Bulletin of Zoology **51(1),**87-95.

Chen IS, Tan HH. 2005. A new species of freshwater goby (Teleostei: Gobiidae: Stiphodon) from Pulau Tioman, Pahang, Peninsular Malaysia. The Raffles Bulletin of Zoology **53(2),** 237-242.

Chen IS, Fang LS. 2006. A new species of Rhinogobius (Teleostei: Gobiidae) from the Hanjiang Basin in Guangdong Province, China. Ichthyological Reseach**53(3),** 247 – 253.

Chen IS, MillerPJ. 2008. Two new freshwater gobies of genus Rhinogobius (Teleostei: Gobiidae) in southern China around the northern region of the South China Sea. The Raffles Bulletin of Zoology **19,** 225-232.

Conservation International Philippines, Department of Environment and Natural Resources-Protected Areas and Wildlife Bureau, Haribon Foundation for the Conservation of Nature. 2006. Priority Sites for Conservation in the Philippines: Key Biodiversity Areas. Quezon City, Philippines: Conservation International Philippines. Published online at http://www.conservation.org/global/philippines.

Conservation International.2013. Biological diversity in the Philippines. Retrieved from http://www.eoearth.org/view/article/1506.48

Department of Environment and Natural Resources – Protected Areas and Wildlife Bureau and Haribon Foundation. 2006. Retrievedat www.bmb.gov.ph.

Devi R, Boguskaya N. 2009.Rhinogobius brunneus. The IUCN Red List of Threatened Species 2009: e.T166994A6296768. Downloaded on 05 April 2018 http://dx.doi.org/10.2305/IUCN.UK.20092.RLTS.T166994A6296768.en

Froese R, Pauly D (Eds.). 2006. FishBase. World Wide Web electronic publication. www.fishbase.org,version03/2006

Greenfield DW, Jewett SL. 2011. Eviota rubriceps, a new goby from the South-western Pacific Ocean, with comments on E. mikiae and E. raja (Teleostei: Gobiidae). Zootaxa **3134,**53–62.

Herre AW. 1927. Gobies of the Philippines and the China Sea. Monographs of the Bureau of Science 23. Philippine Bureau of Science, Manila.

Hossain MY, Ohtomi J, Ahmed ZF, Ibrahim AHM, Jasmine S. 2009. Length-weight and morphometric relationships of the tank goby Glossogobius giuris (Hamilton, 1822) (Perciformes: Gobiidae) in the Ganges of Northwestern Bangladesh. Asian Fisheries Science **22(3),** 961-969.

Huang SP, Chen LS. 2007. Three new species of Rhinogobius Gill, 1859 (Teleostei: Gobiidae) from the Hanjiang basin, Southern China. The Raffles Bulletin of Zoology **14,**101-110.

Huckstorf V. 2012. Rhinogobius giurinus. The IUCN Red List of Threatened Species. Version 2014.2. www.iucnredlist.org

Jaafar Z, Larson HK. 2008. A new species of mudskipper, Periophthalmus takita (Teleostei: Gobiidae: Oxudercinae), from Australia, with a key to the genus. Zoological Science **25(9),**946 – 952.

Kannan KK, Sureshkumar L, Ranjith KK, Joshi, Madan MS, John S. 2013. First record of the two-stripe goby, Valenciennea helsdingenii (Gobiidae, Gobiiformes) from the southeast coast of India. ZooKeys **323,**91–97.

Keith P, Vigneux E, Bosc P. 1999. Atlas des poissons et des crustacés d'eau douce de La Réunion. Muséum national d'Histoire naturelle, Paris. Patrimoines Naturels **39,** 136.

Keith P, Lord C, Lorion J, Watanabe S, Tsukamoto K, Couloux A, Dettai A. 2010. Phylogeny and biogeography of Sicydiinae (Teleostei: Gobiidae) inferred from mitochondrial and nuclear genes. Marine Biology **158 (2),**311 – 326.

Kok HM, Blaber SJM. 1976. A new freshwater goby (Teleostei: Gobiidae) from the Pongolo floodplain, Zululand, South Africa. Zoologica Africana **12(1),** 163-168.

Kottelat M, Whitten AJ,Kartikasari SN, Wirjoatmodjo S. 1993. Freshwater fishes of Western Indonesia and Sulawesi. Periplus Editions, Hong Kong. 221 p.

Larson H. 2012. Glossogobius bicirrhosus. The IUCN Red List of Threatened Species. Version 2014.2. www.iucnredlist.org

Liu ZJ, Cordes JF. 2004. DNA marker technologies and their applications in aquaculture genetics; a review. Aquaculture **238,**1-37. www.elsevier.com/locate/aquaonline

Masuda H, Amaoka K, Araga C, Uyeno T, Yoshino T. 1984. The fishes of the Japanese Archipelago. Tokai University Press, Tokyo, Japan.

Miller PJ, Wongrat P. 1979. A new goby (Teleostei: Gobiidae) from the South China Sea and its significance for gobioid classification. Zoological Journal of the Linnean Society **67(3),**239 – 257.

Myers P, Espinosa C, Parr CS, Jones T, Hammond GS, Dewey A. 2012. The Animal Diversity Web (online). Available at http://animaldiversity.org

Nguyen VH. 2005. Freshwater fishes of Vietnam. Agricultural Publishing House **3.**

Rasmussen RS, Morrissey M. 2008. DNA-Based Methods for the Identification of Commercial Fish and Seafood Species. Comprehensive Reviews in Food Science and Food Safety. Volume 7, Issue 3 June 2008 Pages 280–295.

Parenti LR, Maciolek JA. 1993. New Sicydiinae gobies from Ponape and Palau, Micronesia, with comments on systematics of the subfamily Sicydiinae (Teleostei: Gobiidae). Bulletin of Marine Science **53(3),** 945–972. University of Miami – Rosenstiel School of Marine and Atmospheric Science.

Samonte IE. 2003. Phylogenetic relationships of endemic Lake Victoria cichlids inferred from mitochondrial and nuclear DNA Data. An unpublished dissertation. .Eberhard Karls Universitat Tuebingen.

Sanda R, Kovacic M. 2009. Freshwater gobies in the Adriatic drainage basin of the Western Balkans. Annales – Series Historia Naturalis **19,** 1-10.

Sato A, Takezaki N, Tichy H, Figueroa F, Mayer WE, Klein J. 2003. Origin and Speciation of Haplochromine Fishes in East African Crater Lakes Investigated by the Analysis of Their mtDNA, Mhc Genes, and SINEs. Molecular Biology and Evolution **20(9),**1448–1462. 2003.

Serov DV, Nezdoliy VK, Pavlov DS. 2006. The Freshwater Fishes of Central Vietnam. KMK Scientific Press Ltd., Moscow, Nha Trang.

Shibukawa K, Aonuma Y. 2007. Three new species of the deep-dwelling goby genus Obliquogobius (Perciformes: Gobiidae: Gobiinae) from Japan, with comments on the limits of the genus. Bulletin of the National Museum of Nature and Science, p 137–152.

Suk HY, Choe JC.2002a. Females prefer males with larger first dorsal fins in the common freshwater goby. Journal of Fish Biology **61,**899-914.

Talwar PK, Jhingran AG. 1991. Inland fishes of India and adjacent countries. Volume **2,** A.A. Balkema, Rotterdam.

UPLB Limnological Research Station. 2011. Freshwater fishes in Southern Luzon. University of the Philippines Los Baños, 4031 College, Laguna, Philippines p 1-35.

Vedra SA, Ocampo PP.2013. Morphology of Rhyacichthys aspro (Valenciennes 1837) in Mandulog river system, Iligan city, Northern Mindanao, Philipines. International Journal of Research in BioSciences **2(2),**93-105.

Vedra SA, Ocampo PP, De Lara AV, Rebancos CM, Pacardo EP, Briones NM. 2013. Indigenous goby population in Mandulog River System and its conservation by communities in Iligan City, Philippines. Journal of Environmental Science and Management **16(2),** 11-18.

Walberg MW, Clayton DA.1981.Nucleic Acids Research**9(1981),** 5411-5421.

Ward RD, Zemlak TS, Innes BH, Last PR, Hebert PDN. 2005. DNA barcoding of Australia's fish species. Philosophical Transactions of the Royal Society B **360,** 1847–1857.

Xie S, Cui Y, Li Z. 2001. Small fish communities in two regions of the Liangzi Lake, China, with or without submersed macrophytes. Journal of Applied Ichthyology **17,**89-92.

Xie ZY, Du JZ, Chen XQ, Wang YX, Murray BW. 2006. The significance of mitochondria control region (D-Loop) in intraspecific genetic differentiation of fish. Hereditas (Beijing)**28,**362-368.