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Research Article

NEW RECORD OF THE GENUS *EUCLEA* (LEPIDOPTERA: LIMACOIDIDAE) FROM SOUTH INDIA REVEALED BY DNA BARCODING

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ABSTRACT

The genus *Euclea* (Lepidoptera: Limacoididae) is newly recorded from South India by using DNA barcoding analysis. The larvae of *Euclea* were collected from nearby paddy field at Viralimalai, Tiruchirappalli, Tamil Nadu. The genomic DNA was isolated from the larval heamolymph and the cytochrome oxidase subunit 1 (COI) gene and was successfully amplified. The amplified PCR product was visualized by Agarose gel electrophoresis and the size was found to be approximately 500 bp. Further, the amplified product of the COI gene was purified and sequenced. The sequence analysis revealed that the *Euclea* larvae collected from the study site are closely related to *Euclea norba*. The phylogenetic analysis provides the data to understand the distribution of this species in India and reveals the phylogeny of this genus *Euclea*.

Keywords: Heamolymph, Genomic DNA, *Euclea norba,* Cytochrome oxidase subunit-1, Polymerase Chain Reaction.

INTRODUCTION

Insects are one of the most diverse and successful groups of animals on the planet. Insects thus have great potential for understanding ecosystems and to act as measures of ecosystem health, but the incompleteness of knowledge and the limitation of resources, increase the difficulty of working on insect biodiversity (Wiggins, 1983; Nigel, 2007). There are 29 orders of insects present in the world (Arnett and Jacques, 1981). Every year new species of insects have been discovered by entomologist based on traditional taxonomic data, including colour, morphometric and biochemical markers (Pashley, 1989; Dyer, 1891). However, taxonomic tools are often laborious, time consuming and require considerable skills. Therefore the molecular markers are powerful tools for identifying the insect; prepare molecular catalogue and systematic analysis (Hoy, 2003). It is also evident that molecular identification of insects may be useful in supplementing conventional taxonomic identification.

The mitochondrial genes such as Cytochrome oxidase 1 and 2 (namely CO1 & COII), Internal transcribed spacer (ITS) and 28 S rDNA were widely used as an informative molecular markers for identification of insects other than the study of biodiversity, including population genetics (Avise, 1994; Roehrdanz, 1995). The Cytochrome Oxidase gene is approximately 1.5 kbp in length, encoding a three polypeptide subunit. It is the terminal enzyme of the respiratory chain. The COI gene was already explored for molecular phylogenetic analysis due to its valuable utility in species-level identification and studying the genetic polymorphism of various insects from different orders as follows: Diptera species (Clary and

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Wolstenholme, 1985; Smith, 2006; Wells and Williams 2007; Anbalagan *et al*., 2012), Lepidoptera (Caterino *et al.,* 2000; Hebert, 2004; Hajibabaei, 2006), Hymenoptera (Fisher, 2008; Smith, 2008), Coleoptera (Greenstone, 2005). The present study was aimed to identify the larvae of insect by using COI bar-coding gene.

MATERIAL AND METHODS

Insect collection

The larvae of unknown insects were collected from the nearby paddy field at Viralimalai (Lat: 10° 60'. 711" N, Long: 78° 54'. 057" E) and the insects were transported to Insect molecular Biology Laboratory, Department of Environmental Biotechnology, Bharathidasan University, Tiruchirappalli - 620 024, Tamil Nadu, India for further process.

DNA isolation

The genomic DNA was isolated using a salting out method described by Miller *et al*. (1988) with slight modification and named the lysis buffer as Hemocyte Lysis Buffer (HLB). This methodology has been successfully utilized for the extraction of genomic DNA from insect gut bacteria (Kannan et al., 2015). The heamolymph was collected by making a slit in prolegs and flowed from the wound without external pressure into an Eppendorf tube which was pre-rinsed with Phenylthiourea to prevent the tyrosinase activity (Pakkianathan *et al*., 2012). The hamolymph sample was centrifuged at 10,000 rpm at room temperature (RT) for 5 minutes. The pellet was mixed with 300 µl of HLB consisted of 10 % SDS (w/v), 1 M Tris-HCl, 0.5 % of EDTA and 4 M Nacl. After mixing, the mixture was incubated with RNase (10mg/mL) for 5 minutes. Then 100 µl of saturated 6 M NaCl and 200 µl of Chloroform was added and shaken vigorously for 30 seconds, followed by centrifugation for 10, 000 RPM for 10 minutes. The supernatant was transferred to a fresh 1.5 ml Eppendorf tube. An equal volume of absolute ethanol was added carefully down the wall of the tube. The mixture was centrifuged again at 10,000 RPM for 5 min and the supernatant discarded. Subsequently, the precipitated DNA was pipetted out to a new Eppendorf tube and washed twice with 70 % ethanol by centrifugation at 5000 RPM for 5 minutes. The pellet was air dried at room temperature for 10 minutes. Finally the pellet was dissolved in 50-100 µl of TE buffer (pH 8) and stored at -80°C freezer until further use.

Quantitative and qualitative analysis of isolated genomic DNA

The isolated DNA was subjected to spectrophotometric analysis (Ultrospec 2100, Amersham Bioscience, Hong Kong) to identify the quality and quantity. DNA purity was determined from the ratios 260/280 nm (an indicator of protein contamination) and 260/230nm (indicator of organic solvent residues). The size and intactness of the isolated DNA was checked by agarose gel electrophoresis. The isolated DNA was loaded on 1% agarose gel stained with ethidium bromide (1 µg/µl) and run for 30 min at 60 V. For image acquisition, a LAB India gel documentation system (Infinity, UK) was used. The isolated genomic DNA size was determined by using a 1kb DNA ladder (Biotool, Spain).

Polymerase chain reaction and sequencing

The Cytochrome oxidase subunit I (COI) gene was amplified using PCR with following reaction volume and conditions. The reaction volume (30 µL) containing 15 µL of 2X Prime Taq, 1.5 µL both primers COI-F-C1-J-1751(5′-GGATCA CCTGATATAGCATTCCC-3′) and COI-R -C1-N-2191 (5′-CCAGGTAAAATTAAAATATAA A CTTC- 3), 8 µL of Milli Q water and 4 µL of extracted DNA as a template. Reaction conditions contain initial denaturation step of 3 min at 94ºC, then denaturation step of 30 Sec at 94ºC, an annealing step of 1 min at 50ºC and an extension step of 2 min at 72ºC, followed by 34 cycles. The last cycle included an extended elongation step of 7min at 72ºC. PCR products were analyzed by electrophoresis. The sizes of the amplified fragments were estimated by comparison with a 1kb DNA Ladder used as the molecular weight standard. The desired band was cut and eluted by using a PCR product purification Kit (RBC Inc. India). The purified product was sequenced directly on an automated sequencer (ABI PRISM 3730XL DNA Analyzer, Applied Biosystems, Eurofins, # 183, 1st floor, Gayathri Tech Park, EPIP 2nd Phase, Whitefield, Bangalore - 560 066, India). The sequence generated in this study was deposited at the National Center for Biotechnology Information (NCBI), USA.

Phylogenetic analysis of COI gene

Neighbor-joining (NJ) and maximum parsimony analyses was used for identifying species groups as applied by Hebert *et al*. (2003, 2004) and Sha *et al*., (2006). NJ trees were constructed using the Kimura 2-parameter (K2P) model (Kimura, 1980; Saitou and Nei, 1987) and pair wise distances were obtained using MEGA version 4.0 (Kumar *et al*., 2008). For comparison with other Limacodids, the nucleotide sequences were downloaded from GenBank (http://www.ncbi.nlm.nih.gov/genbank/), NCBI, USA as follows: Five sequence from the genus *Euclea* (Lepidoptera; Limacodids) (Accession number: GU162240.1; GU162253.1; JQ567725.1; HM894287.1; GU162221.1). In addition, we used three COI sequences of other limacodids as the out group: sequence of three COI sequences (accession number: KF595046.1; KF595047.1; KJ638169.1) of the Genus parsa (Limacodids; Parsa) and two COI sequences (GU162275.1; GU162280.1) of the Genus Euprosterna (Limacodids; Euprosterna).

RESULTS AND DISCUSSION

In the present study, genomic DNA was successfully isolated from the larval heamolymph by using HLB method. The genomic DNA size was estimated approximately at 10kbp with the comparison DNA size marker (Biotool, Spain) (Figure 1, L1). The COI gene was amplified and the product was found to be 500 bp in the 1.5% agarose gel electrophoresis (Figure 1, L2). The CO1 gene was purified and sequenced by the method of Sanger sequencing method (Sanger and Coulson, 1975). The nucleotide BLAST result of the sequence of the COI gene revealed that larvae collected from study site is closely related to *Euclea norba*. Further analysis with other mitochondrial genes needs to be carried out to know the molecular evolution of *Euclea sp* (Mandal et al., 2014). The existence of genus *Euclea* (Order: Lepidoptera; Family: Limacodidae) has not yet discovered in India. The larvae of *Euclea* species was typically green and the final instar larval size was estimated using scaling approximately 2cm in length (Wagner, 2005). The life cycle of insect *Euclea sp* consists: egg, larvae, pupae and adult. DNA barcoding analysis suggest that the existing morphological and behavioral data of *Euclea* species. The larvae of insect *Euclea sp.* cause skin allergy effect (Figure 2). Great distribution of insect Limacodids was reported from throughout USA, Texas, Florida, Southern Asia, China and Taiwan (Dyar, 1891; Holloway 1986; Solovyev 2011).

The phylogenetic trees of the 10 limacodids resulting from phylogenetic analysis are presented in Figure 3 and 4. The COI (mitochondrial DNA) gene based phylogenetic tree analysis was more useful to study the evolutionary difference and distance among the species. The Neighbor (NJ) tree and Maximum parsimony (MP) tree commonly shared two monophyletic clades. In the NJ and MP, each shared 5 species. The NJ tree clade 1 formed two groups, in which the first group consisted of three species *P. pygmy, P. media* and *Euclea sp.* and the second group consist two species *(Euprosterna wemilleri).* The NJ and MP tree showed that other *Euclea* sp is distantly related to *Euclea* sp. During, Initial submission of COI sequence at NCBI, it was shown similarity with *Euclea norba* and however the NJ tree showed association with P. *pygmy* and *P. media* in both NJ and MP tree (Figure 3 and 4). Hence, the result indicates that *Euclea* sp. might be close to either the genus *Parsa* or *Euclea* sp and the morphological character (colour and size of larvae) agree to be more similar to *Euclea sp*. Several species of Limacodids are causing skin allergies in America, South Asia, China and Taiwan (Hossler, 2009).

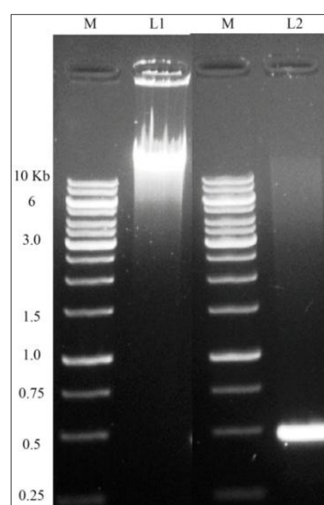

Figure 1. Electrophoretic analysis of genomic DNA isolated from larval heamolymph of *Euclea*. **M** stands for 1 Kbp DNA ladder (Gene Ruler ™ DNA ladder Mix), **L1** stands for genomic DNA and **L2** stands for PCR product of COI gene.

Figure 2. The larvae of *Euclea sp.*

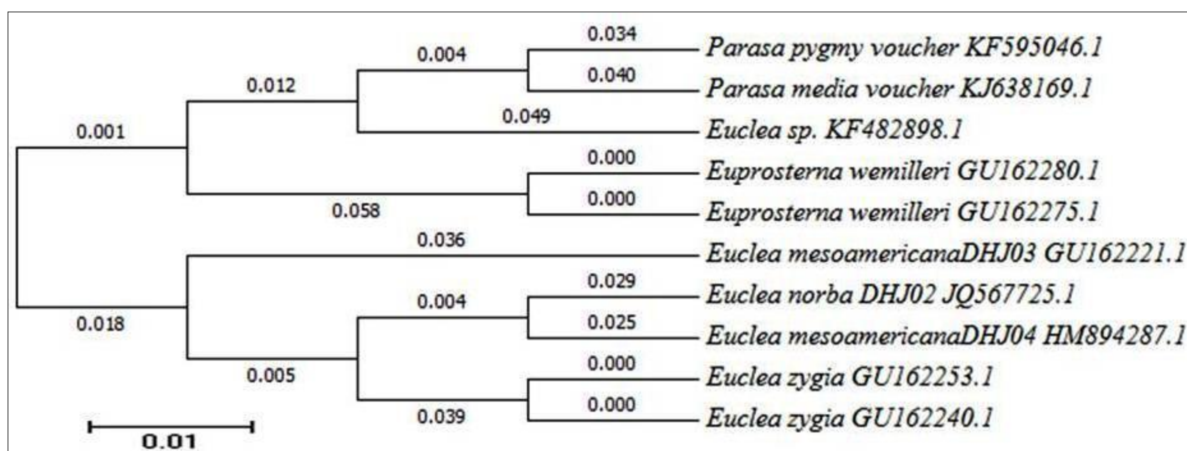

Figure 3. Phylogenetic tree based on COI sequences generated by Neighbor Joining (NJ) method.

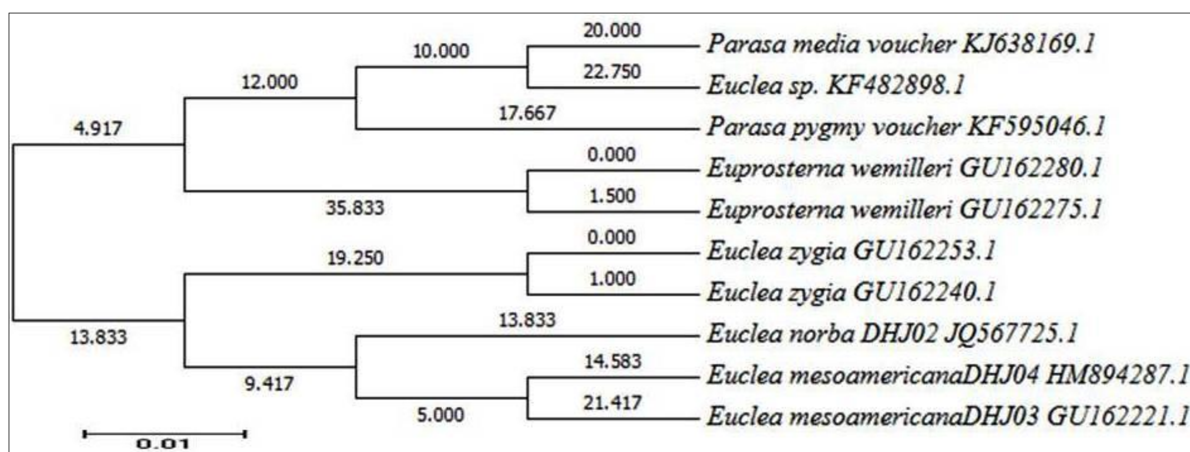

Figure 4. Phylogenetic tree based on COI gene sequences generated by Maximum parsimony (MP) method.

CONCLUSION

DNA barcoding analysis effectively identified the larvae of *Euclea* collected from south India to study the morphological description, life cycle pattern and ecological importance in future.

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REFERENCE

Anbalagan, S., Bharathiraja, C., Pandiarajan, J., Dinakaran, S. and Krishnan, M., 2012. Use of random amplified polymorphic DNA (RAPD) to study genetic diversity within a population of blackfly, *Simulium gravelyi* from Palni hills, peninsular India. *Biologia.,* 67: 1195-1203.

Arnett, R.H. and Jacques, R.L., 1981. Simon and Schuster's Guide to Insects. Simon and Schuster Inc. New York.

Avise, J.C., Arnold, J. and Ball, R.M., 1987. Intraspecific phylogeography the mitochondrial-DNA bridge between population genetics and systematics. *Ann. Rev. Ecol. Evol. Sys*., 18: 489-522.

Caterino, M.S. and Sperling, F.A.H., 1999. Papilio phylogeny based on mitochondrial cytochrome oxidase I and II genes. *Mol. Phyl. Evol.,* 11:122-137.

Clary, D.O. and Wolstenholme, D.R., 1985. The mitochondrial DNA molecule of *Drosophila yakuba*: nucleotide sequence, gene organization, and genetic code. *J. Mol. Evol.*, 22: 252-271.

Dyar, H.G., 1891. A revision of the species of euclea, parasa and packardia, with notes on adoneta, monoleucaand *Varina ornata* Neum. *Am. Entomol. Soc.,*18: 149-158.

Fisher, B.L. and Smith, M.A., 2008. A revision of Malagasy species of *Anochetus Mayr* and *Odontomachus Latreille* (Hymenoptera: Formicidae). *PLoS ONE.,* 3: e1787.

Greenstone, M.H., Rowley, D.L., Heimbach, U., Lundgren, J.G., Pfannenstiel, R,S. and Rehner, S.A., 2005. Barcoding generalist predators by polymerase chain reaction: carabids and spiders. *Mol Ecol.*, 14: 3247-3266.

Hajibabaei, M., Janzen, D.H., Burns, J.M., Hallwachs, W. and Hebert, P.D.N., 2006. DNA barcodes distinguish species of tropical Lepidoptera. *Proc. Natl. Acad. Sc*., 103(4): 968-971.

Hebert, P.D.N., Cywinska, A., Ball, S.L. and DeWaard, J.R., 2003. Biological identifications through DNA barcodes. *Proc. R. Soc. Lond. B.,* 270: 313-321.

Hebert, P.D.N., Penton, E.H., Burns, J.M., Janzen, D.H. and Hallwachs, W., 2004. Ten species in one: DNA barcoding reveals cryptic species in the Neotropical skipper butterfly *Astraptes fulgerator*. *Proc. Natl. Acad. Sci*., 101: 14812-14817.

Holloway, J.D., 1986. The Moths of Borneo: Key to families; families Cossidae, Metarbelidae, Ratardidae, Dudgeoneidae, Epipyropidae and Limacodidae. *Malay. Nat. J.,* 4: 1-166.

Hossler, E.W., 2009. Caterpillars and moths. *Am. Acad. Dermatol.*, Inc. doi:10.1016/j.jaad. 2009.08.061.

Hoy, A.M., 2003. Insect Molecular Genetics: An Introduction to Principles and Applications. Academic Press, New York, pp. 544.

Kimura, M., 1980. A simple method for estimating evolutionary rate of base substitutionsn through comparative studies of nucleotide sequences. *J. Mol. Evol*., 16: 111-120.

Kumar, S., Nei, M., Dudley, J. and Tamura, K., 2008. MEGA: a biologist-centric software for evolutionary analysis of DNA and protein sequences. *Brief Bioinformation.,* 9: 299-306.

Kannan, M., Suganya,T., Arun Prasanna, V., Rameshkumar, N. and Krishnan, M., 2015. An Efficient Method for Extraction of Genomic DNA from Insect Gut Bacteria - Culture dependent. Current Research in Microbiology and Biotechnology., 3 : 550-556.

Mandal, S. D., Chhakchhuak, L., Gurusubramanian, G. and Kumar, N. S.,

2014. Mitochondrial markers for identification and phylogenetic studies in insects-A Review. *DNA Barcodes*, 2:1.

Miller, S.A. and Dykes, D.D. and Polesky, H.F., 1988. A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic. Acids. Res.,* 16(3): 1215.

Nigel, E.S., 2007. Biodiversity: World of insects. *Nature*, 448: 657-658.

Pashley, P., 1989. Host associated differentiation in armyworm (Lepidoptera: Noctuidae): An allozyme and mitochondrial DNA perspective. In: Electrophoretic Studies on Agricultural Pests (Ed. H.D. Loxdale and J. Hollander. *Syst. Assoc. Spec.,* 39, 103-114.

Roehrdanz, R.L., 1995. Amplification of complete insect mitochondrial genome in two easy pieces. *Insect Mol. Biol*. 4: 169-172.

Saitou, N. and Nei, M., 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol*., 4: 406-425.

Sanger, F. and Coulson, A.R., 1975. A rapid method for determining sequences in DNA by primed synthesis with DNA polymerase. *J. Mol. Biol.*, 94 (3): 441-448.

Sha, Z.L., Zhu, C.D., Murphy, R.W., La Salle, J. and Huang, D.W., 2006. Mitochondrial phylogeography of a leafminer parasitoid, *Diglyphus isaea* (Hymenoptera: Eulophidae) in China*. Biol. Control.,* 38: 380-389.

Smith, M.A., Wood, D.M., Janzen, D.H., Hallwachs, W. and Hebert. P.D.N., 2006. DNA barcodes reveal cryptic host-specificity within the presumed polyphagous members of a genus of parasitoid flies (Diptera: Tachinidae). *Proc. Natl. Acad. Sci.*, 103: 3657-3662.

Solovyev, A.V., 2011. New species of the genus *Parasa* (Lepidoptera, Limacodidae) from Southeastern Asia. *Entomol. Rev.*, 91(1): 96-102.

Wells, J.D. and Williams, D.W., 2007. Validation of a DNA based method for identifying Chrysomyinae (Diptera: Calliphoridae) used in a death investigation. *Int. J. Legal Med.,* 121: 1-8.

Wiggins, G.B., 1983. Entomology and society. *Bull. Entomol. Soc. Am.,* 29: 27-29.