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MORPHOLOGICAL IDENTIFICATION AND DNA BARCODING STUDY OF *Labeo calbasu* (Hamilton, 1822) FROM LOWER ANICUT, TAMIL NADU, INDIA

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Abstract

DNA barcoding is a global standard tool in taxonomy based DNA sequences and its usefulness for identification of species. Genus *Labeo* morphologically complicated and it still not fully resolved genus. While, several described species and other species of within genus are very difficult to delimit due to overlapping in morphological characters and complex of color patterns towards *Labeo kontius* and *Labeo fimbriatus* are very similar. With this, we have a complete photographical image for mainly helpful an identification tool for field collection. Now, morphological species identification for within group has been a unique controversial. In this, the next focus specifically a paucity of fish taxonomists and all paving by way of molecular diagnostic tool namely DNA barcode for effective taxa delimitation. Herein, the present phylogenetic analysis of *Labeo calbasu* based on the short standardized segment and 5' region of mtDNA COI gene fragments. Moreover, the present work discussing about to identify species through mtCOI gene to discriminating the wild species *Labeo calbasu* from Cauvery waters (Kollidam river), Lower Anicut, Tamil Nadu. Finally, it is a complement current results provide evidence that mtCOI gene are more effective for rapid and accurate identification of species and certain discrimination were found this species that need for further taxonomic investigation.

Key words: Photographic image, MtCOI gene, Phylogenetic tree and Species identification.

1. Introduction

The delimitation and recognition of fish species *Labeo calbasu* are not only interest for taxonomy and systematics, but also an imperative management of fisheries for authentication of food resources (Rasmussen *et al.*, 2009). Like, it is an important food fish having wider distribution in many countries like India, Pakistan, Myanmar, Thailand, Yamuna, South China (Reddy, 1990) and Bangladesh (Alam and Islam, 2005). However, due to some complexity and limitations of morphological characters was used is very necessity of traditional taxonomy. Besides, diverse difficulties in relying primarily on morphology when attempting to identify fishes during various stages of their development. Even, when intact adult specimens are available the morphological characters used to discern species can be subtle identification are difficult, even for trained

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taxonomists (Ward *et al.,* 2009). In addition, the taxonomy of *Labeo* genus as a whole has remained somewhat confused (Jayaram and Dhas, 2000). Approximately 105 extant species in *Labeo* are presently recognized, of which 69 occur in Africa and 36 in Asia. Revisions of members from Africa and Indian region (Jayaram and Dhas, 2000) have been made on morphological grounds. However, upto date the molecular genetic evidence for revision of the genus has not been evaluated and also genetic relationships between *L. rohita* (rohu) and other species of *Labeo* remain unknown. Hence, there is a need to way for molecular diagnostic tools such as DNA barcoding for effectual taxa delimitation to investigate the phylogeny of *Labeo* group of fishes. It is a well recognized DNA-based identification system or commonly known as DNA barcoding (Hebert *et al.,* 2003), can aid the resolution of the vast diversity of life with its millions of species (Tautz *et al.,* 2003). It has a great deal to offer especially in the provision of tools enabling unequivocal specimen identification and stock structure assessment (Ward, 2000). Currently, DNA barcode is a cost-effective option for identification of species, in some situations this will be increasingly as reference libraries for assembled and analytical protocols are simplified (Hajibabaei *et al.,* 2005). Furthermore, the DNA barcoding supposedly would be a fast, efficient and globally accessible method for delimiting and identifying new species (Hebert *et al.,* 2003). Thus, the mitochondrial cytochrome *c* oxidase I (mtCOI) gene was chosen as the standard barcoding one because it shows a conserved amino acid sequence that facilitates the design of universal primers are applicable to a diverse group of organisms (Hebert *et al.,* 2003 & 2004).

In addition, morphological examinations of variation within species, identification of stock structures of fishes, phylogenetic variations of within and between species (Billington, 2003). Furthermore, it may act as conservation and rehabilitation of fish species, widely used owing their slowest mutation and lower substitution rates. It encodes for a subunit of cytochrome *c* oxidase an enzymatic protein complex and absolutely required for aerobic metabolism, phylogenetic reconstructions and availability of related sequences from publicly curate databases, maternal inheritance of the mitochondrial genome and reduced occurrence of mitochondrial gene recombination (Simon *et al.,* 2006). Like the two basic assumptions that underpin the barcoding methodology is monophyly of the species with respect to molecular markers and intra-specific genetic divergence. Further, the genetic difference among species, thus justifying use species divergence thresholds to assign the individuals for correct species based genetic divergence. However, it has proved a very useful for accelerating species recognition, biodiversity assessment and conservation efforts, stock management, ecosystem monitoring and phylogeographic studies (Nwani *et al.,* 2011). In this framework, the use of DNA barcodes facilitates identification to a high degree of certainty of various life-forms including larvae, carcass fragments, protected species and damaged specimens (Ward *et al.,* 2005). Additionally, its efficiency has also been proved in recent studies of freshwater fish (Nwani *et al.,* 2011). At present, the indigenous fish populations of *L. calbasu* in Kollidam river, Lower Anicut, Tamil Nadu are threatened both biological and physical factors, *i.e.,* introduced new species, pollution, overharvesting and habitat degradation. Unfortunately, very inadequate information on this species in Lower Anicut. For instance, several situations of the genetic data are the best way to determine whether the species is worth as a special protection under Endangered Species Act (ESA) or other form of conservation status (Matoso *et al.,* 2004). Following, as it allows calculated decisions on the best course of action to be in use for protection and conservation (Leuzzi *et al.,* 2004) as well as managing for different stocks (Salini *et al.,* 2006). As well, the present research work is a part of the Ph.D. thesis to investigate molecular based systematic approach of this species. Not only prove the potential use of traditional taxonomy of freshwater fish but also will help further in easy identification of the studied species (Bhattacharjee *et al.,* 2012).

2. Materials and Methods

Sampling area and Collection of samples

Lower Anicut (Kollidam river) is one of the major freshwater fishery resources within the northern region of Tamil Nadu (11° 15' N latitude and 79° 30' E longitude) which is selected for the present study (Figure 1). The river flows from west to east forming the northern boundary of this block. It is a main commercial landing centre for fish fauna along with varieties of fin and shell fishes, where the Tamil Nadu State Fisheries Department has the sole authority for landing and marketing of fishes throughout the year. Among the varied fish fauna landed, the family of Cyprinidae is one of the dominated fisheries of this region. Live specimens of *L. calbasu* were collected from the landing centre at Lower Anicut, Tamil Nadu during January 2012 - March 2012. Fifteen individuals of fresh specimens were used for DNA barcoding study. After collection, specimens were weighed in total length (mm) was measured with the help of measuring board. Total weight (g) was measured with an electronic balance (DIGI' Arts maximum = 1000 g to d = 0.5 g). Specimens were identified morphologically using scientific literature relevant to the group with original descriptions by Talwar and Jhingran (1991). After identification, specimens was photographed in all morphological body parts alive (left-hand side) prior to tissue sampling. Consequently, the tissue samples (*i.e.*, caudal fin) were collected and released their living place. Afterwards, samples were stored in sterile eppendorf tubes containing 95 % ethanol, sealed with parafilm kept at room temperature until further analysis. Moreover, the present approach of sampling has been made to gather complete information on systematic aspects of morphological (photographic identification) and cytogenetically.

Illustrative Photographical Image Identification

The wild populations of *L. calbasu* were employed by invasive photographic techniques. Colour patterns of body and fins which are clearly focused for identification of images. *L. calbasu* in order to obtain more precise images of the selected individual, using a digital camera (SAMSUNG-PL 20 with 5x level; Lens focal length: 4.9-24.5; mega pixel 14.2 using Adobe Photoshop CS3) by Samsung manufacturer. Photographs were taken perpendicular to the subject were only used when the fish with all types of fins were fully extended. Additionally, the resulting of the high-resolution images was used to extrapolate the morphological image identification.

Genomic DNA extraction, PCR amplification and Sequencing

Genomic DNA was extracted from the individuals using standardized salting-out procedure with some modifications (Sambrook *et al.*, 1998). Polymerase chain reaction was performed to amplify 700 bp fragment of mitochondrial cytochrome *c* oxidase I (mtCOI) gene was using a universal specific primer, following: Forward Fish F1 5'-TCAACCAACCACAAAGACATTGGCAC-3' and Reverse Fish R1 5'–TAGACTTCTGGGTGGCCAAAGAATCA – 3' (Ward *et al.*, 2005). A 25 µl PCR mixture contained 1.0 - 2.0 µl of DNA template, 2.5 µl 10x$Mgcl_2$ buffer, 1.0 µl primer mix, 2.0 µl dNTP, 0.5 µl Taq DNA polymerase and 18.0 µl deionised ultra-pure water. PCR reaction was performed in the following condition (TechGene^{TM}) and thermal cycling profile are as follows: initial denaturation at 94 ºC for 5 minutes denaturation 35 cycles at 94 ºC for 30s, annealing at 54 ºC for 30s, extension at 72 ºC for 1minutes and final extension at 72 ºC for 10 minutes. Prior to sequencing, PCR products were check visualized for quality and length conformity on 1.5 % using agarose gel electrophoresis. Molecular weight was also checked using molecular weight markers (100 bp ladder).

Phylogenetic inference and Statistical analyses

Bidirectional sequences of the amplified COI gene products were purified by automated capillary sequencer (ABI 3100 PE). Nucleotide sequences were aligned and edited based on

chromatogram inspection using FinchTV 1.4. Sequences trimmed according to the translated vertebrate mitochondrial amino acid code program namely MEGA ver. 5.0 (Kumar *et al.,* 2011). As a result of obtained the similar sequences from National Center for Biotechnology Information (NCBI), Barcode of Life Database (BOLD) and Basic Local Alignment Search Tool (BLAST) program was performed. Based on the percentages of similarity, the species was confirmed along within and between species sequences was selected with high similarity phylogenetic analysis. Sequences have been deposited to NCBI GenBank and accession numbers by barcodes. Phylogenetic trees were constructed using to provide a graphic representation of species divergence through Neighbor Joining (NJ) algorithms were implemented by MEGA ver. 5.0. Sequence analyses were conducted using pair-wise genetic distance and nucleotide composition analysis, disparity index and tajima's neutrality test. Pair-wise distances were calculated based on mtCOI gene sequence analyses using Kimura 2-parameter model (Kimura, 1980). All the nucleotide sequence analysis were treated concerning 637 codon positions and 1^{st} + 2^{nd} + 3^{rd} + non coding. The disparity index and computing evolutionary distances that account on the nucleotide frequency bias for correcting multiple patterns of nucleotide substitutions has remained throughout the evolutionary history of examined sequences. However, the test conducted using pair sequences and it does not require knowledge of the actual patterns of substitution. In addition, the *P*-value of the disparity index for rejecting the null hypothesis of homogenous pattern was assessed by MEGA 5.0. The assuming neutrality evidence of a population expansion was also tested using Tajima's D (Tajima, 1983) as implemented by MEGA ver. 5.0. The Tajima's D statistic is a selective neutrality test decides whether the mean number of differences between pairs DNA sequence is compatible with observed number of segregating sites in a sample. Significantly the negative values of the statistics indicate an excess of new mutation in relative to equilibrium expectations on the basis number of segregating sites.

3. Results and Discussion

Key to species

Dorsal fin rays 16-18. Barbels two pairs and fins are black; pectoral fins as long as head length; mouth distinctively inferior inside (Talwar and Jhingran, 1991).

Distinguishing characters

Body stout and rather than deep. Head fairly large and conical, its length less than body depth. Snout depressed and fairly pointed, devoid of lateral lobe. No pores on snout. Eyes are moderate, visible from underside of head, the diameter about 3.3 times in head. Mouth inferior, lips thick and conspicuously fringed, both lips with a distinct inner fold. Barbels two pairs, (maxillary and mandibular) maxillary pair longer than mandibular pair (Rahman, 2005). Dorsal fin with a fairly long base, inserted midway between snout tip and base of caudal fin. Caudal fin deeply forked. Scales are moderate; lateral line with 40-44 scales; lateral transverse scale-rows 5-6 between lateral line and pelvic fin base; there are 20 rows of scales before dorsal fin and 22 rows circumference of the caudal peduncle. Pre dorsal scales 19-21 was occur (Talwar and Jhingran, 1991).

Fin formula

D. iii-iv 13-16; A. ii-iii 5; P. i 16-18; V. i 8 (Talwar and Jhingran, 1991).

Colour

In life, blackish-green, lighter below; flanks buff pink or with scarlet spots with dark edges which may form stripes. Fins black; upper lobe of caudal fin usually tipped with white (Talwar and Jhingran, 1991).

Photographical (morphological) image identification

The present study can be distinguished of the photographical image identification for *L. calbasu*. Herein, present morphological characters results suggest there are two unbranched and fourteen branched rays were covered in dorsal fin; one unbranched and eight branched rays in ventral fin (Figure 3a and 3b). Whereas, one unbranched and sixteen branched rays in pectoral fin and two unbranched and six branched rays in anal fin. Furthermore, the upper lobe of caudal fin have two unbranched and nine branched rays whereas two unbranched and nine branched rays in lower lobe (Figure 4a and 4b). The mouth structure of *L. calbasu* was clearly indicated as a bottom feeder. Barbels are covered both of rostral and maxillary region and rostral barbel longer than maxillary. The size of rostral barbel nearly in 1.4 cm and 0.8 cm in maxillary (Figure 2). Five pairs of branchiostegal rays and four pairs of gill arches in each side of the operculum. Each gill arch contains 67 to 71 gill rakers. A pair of external nostrils and a forked caudal fin rays are clearly visible. Lateral lines are straight and forty one scales are arranged in the middle region of the body starting from operculum to caudal peduncle with flank dark pinkish cycloid scales around in lateral line (Figure 2). In the present result, we suggested that the morpho-taxonomical observation, no more spines are covered all the fins of *L. calbasu*.

DNA barcoding

Isolated genomic DNA (Figure 5a) and PCR amplification mtCOI gene bands displayed in Figure 5b. From the analysis it was confirmed the mtCOI universal primers shows single clear band. However, all individuals of *L. calbasu* show a good amplification with high specificity comparing with molecular marker (1000 bp ladder) product size showed approximately 700 bp. Four nucleotides were colored with four specific labels (Adenine Green, Guanine Black, Thymine Red and Cytosine Blue). All nucleotide sequences were translated into amino acid sequences to check the translation efficiency. The present mtCOI sequences showed 99 % identity (96% query coverage) compared to other *Labeo* species through NCBI databases. Based on the similarity sequence analysis the present sequences confirmed with compared to NCBI sequences as the species confirmed as *Labeo calbasu.* The mtCOI sequences were submitted to GenBank with unique accession number (KC508502, KC508503 and KC508504).

Phylogenetic tree and Statistical analyses

The present results suggest the phylogenetic tree (Figure 6) shows relationship among the species of *Labeo*. The tree roots without out groups on the node indicate the bootstrap support indicates genetic distance. However, the evolutionary history was inferred using Neighbor-Joining (NJ) algorithm (Saitou and Nei, 1987). The percentage of replicate trees in which associated taxa clustered together in bootstrap test (1000 replicates) are shown in next branches (Felsenstein, 1985). The phylogenetic tree is drawn to scale with branch lengths in same units and evolutionary distances used to infer the phylogenetic tree. Evolutionary distances (ED) were computed using *P*-distance method (Nei and Kumar, 2000) and the unit number of base differences per site. The analysis involved for 17 nucleotide sequences with codon positions included were 1^{st} + 2^{nd} + 3^{rd}. All position containing gaps and missing data were eliminated by common deletion method and evolutionary analysis was conducted by MEGA 5.0 (Kumar *et al.,* 2011). The NCBI gene sequence of *L. calbasu* (JQ713848) was closely related to present species *L. calbasu* (KC508503), (KC508504) and *L. yunnanensis* were highly distance compare with first present studied individuals of *L. calbasu* (KC508502). The other sequences *L. calbasu* (JQ713848) and *L. vulgaris* showed high genetic distance among nucleotides. Presently, the pair-wise genetic distance referred as the number of base substitution per site from seventeen sequences were shown in Table 1. All position containing gaps and missing data were eliminated by common deletion method. The evolutionary

analysis was conducted by MEGA 5.0. Furthermore, the maximum level base differences in *L. ariza* and *L. bogus* with minimum level sequence differences between *L. calbasu* (JQ713848) and *L. calbasu* (KC508504). Besides, the base composition bias differences per site are shown in Table 2. In addition, when the substitution patterns are homogenous nature among lineages, the compositional distance will correlate with the number of differences between sequences. Hence, the present study focus the species *L. horie* and *L. yunnanensis* were maximum and minimum compositional distances between *L. calbasu* (JQ713848) and *L. calbasu* (KC508504). The estimates net base composition bias disparity index for between sequences was shown in Table 3. Values are quarter and zero with large differences in base composition biases based on evolutionary divergence between sequences and by chance alone. In this study *L. yunnanensis*, *L. barbatus*, *L. horie* and *L. yunnanensis* were showed maximum and minimal level disparity index between *L. calbasu* (JQ713848) and *L. calbasu* (KC508504). Moreover, the Tajima's D neutrality test between species and genus level variations are given shown in Table 4. The calculated Tajima's test statistic value 0.410623 indicates all the seventeen mitochondrial cytochrome *c* oxidase I nucleotide sequence were under less than 1 (neutrality) indicates this species is too less survey in this environment. The statistical value under was estimated using MEGA 5.0. Therefore, all positions containing gaps and missing data were eliminated from complete deletion option. Subsequently, fisheries are vital sources of protein for human, but also play an essential role in aquatic ecosystem. However, less than 40 % of fishery captures in tropical regions that are described at species level (Caddy and Garibaldi, 2000). In grouping, with the current threats that could originate from various sources such as climate change, pollution, habitat destruction, over-exploitation (Hughes *et al.*, 1997) and limited knowledge diversity of fishes implies the species are likely as *L. calbasu* to go endangered moment in very recent years. Besides, this species has become uncertain due to continuous environmental modification and human interventions were affecting feeding and spawning migration which was declining the species population. Therefore, it will be principally described the raises of necessity for further knowledge about fish diversity. Consequently, the *Labeo calbasu*, *L. kontius* and *L. fimbriatus* are morphologically very similar. However, local researchers regard them all are belonging to same species. Further studies need to be done to confirm whether all the species indeed belong to the same genus or differ. Still, based on the genetic distances were observed this study, the genus *Labeo* are most like different species but possibly should be assigned to the same genus. In addition dorsal, anal fins are colourful one and also caudal fins of all species are deeply-forked. In this background, the present research to investigate the species specific identification and delimitation within the genus of *Labeo*. With this, the present mtCOI gene was used as an experimental evidence for identifying species as *L. calbasu* by DNA barcode. Herein, the 5' region of the mtCOI was selected as the basis for DNA barcoding system because the availability of primers aiding its recovery from a broad range of taxa (Hebert *et al.*, 2003). Thus, the barcode based approaches are provided with additional important data for precise identification of this species. According to Ward *et al*. (2005) reported the phylogenetic mtCOI sequences could effectively used for cluster most congeneric and confamilial species. So far, the above information were observed in earlier studies such as Australian fishes (Ward *et al.*, 2005), Canadian freshwater fishes (Hubert *et al.*, 2008), Mexico, Guatemala (Valdez-Moreno *et al.*, 2009) and Cuban freshwater fishes (Lara *et al.*, 2009). Theoretically, the genetic divergence should increase with also increasing taxonomic levels were assessed by DNA barcode. As expected, this was not supported by the present study *i.e.*, comparisons for within species, genera and families gave very similar values, but probably due to very limited sample sizes at the taxonomical cluster analysis. Furthermore, the limited number of individuals in this study did not

permit further inference to be made on the phylogenetic relations.

Presently, the DNA barcode region of entire mtCOI gene sequenced individual with length varying from 500 bp – 650 bp could be discriminated from other species level. However, the present individuals tightly clustered with *L. calbasu* were downloaded from NCBI databases by distance based phylogenetic analysis. Furthermore, the results were simply reflecting the effect of conspecific sequence identity for between individuals and the same clustering pattern was obtained by previous DNA barcoding study, in which the conspecific individuals were clustered in single clade with bootstrap values of 52 - 100 % (Ward *et al.,* 2005). Thus, the nucleotide composition analysis was also congruent nature in the phylogenetic analysis. However, the congeneric level analysis showed significant variations in each position at codon level. Therefore, much variation was identified in 2nd position as codon level was found than 1st and 3rd positions (Table 1). Besides, the present results were not concordant with the previous findings obtained by Ward *et al.* (2005) and also observed the 3rd position showing more nucleotides variation than other two positions. Moreover, some individuals of *L. calbasu* showed variation occurred in the evolutionary scale through phylogenetic analysis. This might be explained the samples of individuals that obtained by single geographical locations. In previous report that the allozyme surveys of marine fishes indicate that typically only about 5 % genetic variance comes from inter-population differentiation. Furthermore, this percentage variation was appreciably higher for freshwater species, around in 20 % on average (Ward *et al.,* 1994). Typically, the phylogenetic trees were constructed by distance based approach showed distinct clades with congeneric species and also the similar congeneric clustering pattern was obtained from the previous study (Ward *et al.*, 2005). However, the pair wise distance analysis between individuals of conspecific species showed no significant divergence in their nucleotide sequences. Like, the present studied individuals showed no genetic distances about the (*L. calbasu*) sequences were obtained from NCBI's database. Although, they showed significant divergence in their nucleotide sequences for between congeneric species that it was sufficiently enough to identify the studied species with respect to geographical location in future. As a result of the mtCOI gene sequences of *Labeo* species evolving under normal rate like in the other species were found. According to Hebert *et al.* (2003) reported that 98% of sequence pairs showed greater than 2% divergence in their study dealing with animal barcode life. However, the present results concordant with the findings obtained by France and Hoover (2002) and Shearer *et al.* (2002) suggested that the mitochondrial evolution is exceptionally low in animals' life. Otherwise, the results suggest that the prospects for extending in mtCOI based identification system. Finally, some studies suggest that the intra-specific divergence comes from phylogeographic distances. Finally, as a results suggest that the intra-specific divergence are rarely greater than 2% and most of the time less than 1 % (Avise, 2000) in some of the higher divergence reflect the simply effect of their origin in past episodes of gene pool fragmentation (Avise and Walker, 1999). In addition, the present studied species also showed a little significant divergence were found about the individuals of *L. calbasu.* Like, the current study referred that the first and second placed individuals showed slightly variations from other *L. calbasu* and also this might be mainly represented that all the individuals are introgressive hybridization nature for this study area. In addition, the present results are also supports in the Tajima's neutrality test for within genus of *L. calbasu* showed a slightly positive value 'D' obtained from a total of 637 nucleotide positions in the final dataset that indicates no such genetic bottleneck events and subsequent genus expansion as described in the following previous works. According to Lijo (2009) observed a negative Tajima 'D' values in population genetic structure of ornamental teleost (*Puntius denisonni*) suggesting a history of recent genetic bottleneck period with subsequent population expansion. Presently the results indicate that an identification system for *Labeo*

genus based on the mtCOI gene will be highly effective. Thus, to assess the exact population structure of *L. calbasu* in Lower Anicut is very essential for next step of the study to use more molecular markers such as D-loop and microsatellite.

Conclusion

Despite the challenges of getting an accurate identification of species, through DNA barcoding to provide a convenient, accurate and valid tool for identification of species and any candidate gene must suit this qualification. Uses of a single, universal gene has many advantages, especially as barcoding applications expand to ecological questions and the identification of illegally imported parts of organisms (Xia *et al.*, 2012). DNA barcode represents a significant move forward for providing the identification tools for wild species of carps in biosecurity situations. In some critical condition, the small numbers of species individuals where DNA barcodes fail to offer an unambiguous identifications, introgression, incomplete lineage, sorting and complex species are mixed. In this moment, the additional data such as Web-based images of live specimens, morphological characters and gene loci can be call upon to resolve these problematic species (Collins *et al.*, 2012). Although intra-and inter-specific genetic divergences, overlapping, tree-based methods can distinguish species in unidentified samples (Smith *et al.*, 2008). On the one hand, the morphological taxonomy cannot give a definite identification. In this context, for claim that it may be a new species based on molecular analysis without species delimitation. An assumed threshold is helpful to expedite discovery of a new species and biodiversity, especially to dealing with little-studied biota, single, uniformity species delimitation to seems arbitrary because the rates of molecular evolution vary widely for within and among lineages (Ivanova *et al.*, 2007). It is suggested that future studies should incorporate the morphometric methods to resolve the taxonomic status of these undetermined species. Besides, the ecologist and taxonomist alike, DNA barcoding will provide a powerful tool for species identification, biodiversity assessments, association of larva, adult morphologies and suggest the occurrence of cryptic species (Mohanty *et al.*, 2013).

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