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DNA barcoding as a molecular diagnostic tool for the species authentification and phylogenetic analysis of *Copera marginipes* (Odonata: Plactycnemidae)

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Abstract

DNA barcoding is a new genomic technology for recognizing and ordering species. The utility of mitochondrial genes for resolving genus level phylogenetic relationships are well known. The present study investgated the utility of DNA barcoding as a molecular diagnostic tool for the species authentification and phylogenetic analysis of a damselfly species *Copera marginipes* using cytochrome oxidase I gene. The partial coding sequence yielded 616 bp sequence and BLAST analysis confirmed its molecular identity as the same species. Phylogenetic analysis confirmed the taxonomic identity of this species as *Copera marginipes* due to sister taxa relationship with the same species reported from Gujarath and Malaysia. The phylogenetic tree says that the ancestors of Copera genus were splitted at an earlier time with *Copera nyansana* and *Copera sikassoensis* were found in one clade and *Copera marginipes* from Kerala and Malaysia in another clade Phylogenetically its nearest member was found to be *Copera nyansana* and *Copera sikassoensis* with respective divergence of 16.02 and 17.18%.

Keywords: placticnemidae, *Copera marginipes*, DNA barcoding, cyochome oxidase I gene

Introduction

The damselfly family, Plactycnemidae is commonly known as 'white legged damselflies' consisting of 42 genera including 400 species. They often found among long grasses bordering brooks and streams. They are characterised by laterally expanded heads with shallow labial cleft and tibiae with long dense spines [2, 6]. Members of this old family can be easily identified by having dilation of the tibiae in males and sometimes in female [7]. The two hind pairs of tibiae are bright orange to dull reddish, moderately dilated and the superior anal appendage are found only one fourth of the length of inferiors. The discoidal cell of wing is elongate with its costal or anterior side is slightly shorter than the basal and its distal end seems to be subacute.

Copera marginipes is characterised by having bronze black colour with yellow lines on thorax, bright yellowish orange legs, transparent wings with brown wing spot and bronze black coloured abdomen in males (Fig.10). Female members are brown coloured thorax, brownish legs, transparent wings with pale brown coloured wing spot and brown coloured abdomen [8]. This species is usually observed in streams. It is widely distributed in Asia and New guinea.

DNA barcoding is a new innovative research in the field of modern systematics. It is the easiest tool for taxonomic identification using a universal standard gene region among different organisms. This technique was first described by Paul Hebert in 2003 and this method provides a unique "barcode" to every organism in the world for easy identification. The main advantage of this technique is the easy diagnosis of species irrespective of their life stages (as larvae, nymph, adult etc), damages and body decay thereby helping for accurate identification and taxonomic relatedeness.

Mitochondrial DNA became a popular phylogenetics tool the for population studies because of its easy isolation, use of restriction enzymes to detect nucleotide differences, development of PCR methodologies and applicability of universal primers for amplification of DNA [1]. The usual methodology involves extracting DNA from any sample and compare those sequenced DNA against the barcode library for identification thus helps to predict the origin, evolution and evolutionary relationships

Materials and Methods

Sample Collection and Preservation

Dragonflies belongs to Libelluidae family were collected from Kasargode (Figure 1). Collections were made by hand sweep netting and random field sampling method was done to cover the entire study area. Identification was done by observing wing venation, colour pattern and genitalia, described in available keys/identification guides. Additional information regarding date of collection, locality etc., about each specimen was also recorded. Each specimen was then placed in a separate collecting bottle, assigned a code number and stored in 70% ethanol until further use. One or more legs were removed for DNA isolation and kept in ethanol until further use.

Fig 1: Damaselfly collecton site (Kasargode: Kangangad) (12.352º N 75.096º E)

DNA extraction, amplification and sequencing

DNA from selected damselfly was extracted from leg using 'Origin DNA Extraction kit'. The obtained DNA was confirmed using 1% agarose gel. About 2ng of DNA was PCR amplified for mitochondrial cytochrome oxidase subunit I (COI) gene using forward primer (5'-TTTTCTACTAACCACAA –3')and reverse primer (5' TTTTCCTCTTTCTTGGG') in Takara PCR thermocycler. The thermo cycler conditions were slightly modified as follows; 1 initial cycle of 5 minute at 95 °C followed by 30 cycles of 95 °C for 10 seconds and 50 °C for 1 minute, 72 °C for 45 seconds. This is followed by a final step of 72 °C for 3 minutes. The obtained PCR product was checked using 2% agarose gel electrophoresis and were sequenced with both the forward and reverse primers using an automated sequencer ABI 3730XL Sangers method. Phylogenetic analysis done by MEGA software [9].

Data Analysis

Mitochondrial COI sequence data for the selected dragonflies was sequenced and submitted in GenBank. The aligned sequences were used for species identification using BLAST. The sequences from GenBank were retrieved and sequences of each species generated from this study were compared and aligned using clustal w.

Result & Discussion

The partial coding sequence of mitochondrial COI gene of *Copera marginipes* collected from Kasaragod (12.5000° N 75.000° E) district yielded a product having 616bp and translated sequence of 205bp. The DNA sequence interpret, representative molecular barcode, conceptual translation product, nucleotide BLAST, peptide BLAST, line diagram and molecular phylogenetic tree were depicted. The sequence was deposited in nucleic acid databases for public accession having NCBI GenBank Accession No. KR149804 and Barcode of Life Data System BIN Cluster ID – BOLD: ABA1480 with Specimen ID – GBMH0650-15.

Fig 2: *Copera marginipes*

The COI sequence of *Copera marginipes* showed bias to nucleotide AT, with following composition of nucleotides T =3 4.6%, C = 15.8%, A = 31.4% and G = 18.3% (Table 1). This high AT content of 66.0% over 34.1% of GC is mainly due to the mutational pressure on a single nucleotide substitution during the evolutionary period of time. The BLAST analysis showed that this species is 100% sequence similar to the same species reported from Netherland (KF369351).

Table 1: Percentage of evolutionary divergence of *Copera marginipes* with its closely related species accessible from NCBI GenBank

Sl. No.	Accession No	Organism	Percentage of divergence
1	KR149804	*Copera marginipes* (Kerala)	
2	KF369351	*Copera marginipes* (Netherland)	0.00
3	KF369352	*Copera nyansana* (Netherland)	16.02
4	KF369353	*Copera sikassoensis* (Netherland)	17.18
5	KT879906	*Elattoneura vittata*	16.02
6	KX890965	*Ophiogomphus smithi*	18.15
7	KX890938	*Ophiogomphus smithi*	18.15
8	KX890940	*Ophiogomphus westfalli*	18.74

The evolutionary history was inferred using the Neighbour-Joining method and the evolutionary history of the taxa was analyzed. The analysis involved 8 nucleotide sequences and the codon positions included were 1st+2nd+3rd+Noncoding. There were a total of 602 positions in the final dataset after eliminating all positions containing gaps and missing data. The phylogentic tree confirmed the taxonomic identity of this species as *Copera marginipes* due to sister taxa relationship with the same species. Phylogenetically this clade is sister to the clade possessing *Copera nyansana* and *Copera silikkassoenis* indicating genus level taxonomy and monophyletic origin (Fig:3). The percentage of divergence table plotted by Maximum Composite Likelihood model confirmed the above statement. The nucleotide sequence showed respective divergence of 16.02-17.18% with *Copera nyansana* and *Copera sikassoensis* (Table 2). The sequence has also submitted for BOLD system, another database to confirm the species authenticity. The analysis showed 99.82-100% sequence similarity to out of 15 different similar matches already reported in BOLD system. The line diagram of this species for the confirmation of above statement is shown in figure: 10f. The close matching BIN of the species was found to be 3% and its average and maximum distance was respectively as 0.88% and 2.5% (BOLD: ABA1480). The average and maximum nucleotide distance to the nearest member was found to be 0.43% (p-distance) and 1.61% (p-distance) respectively (BOLD: ADC5413). The 100% similar species was found to be reported from Gujarat and Malaysia having accession numbers (BOLD: ABA1480 and KF369351).

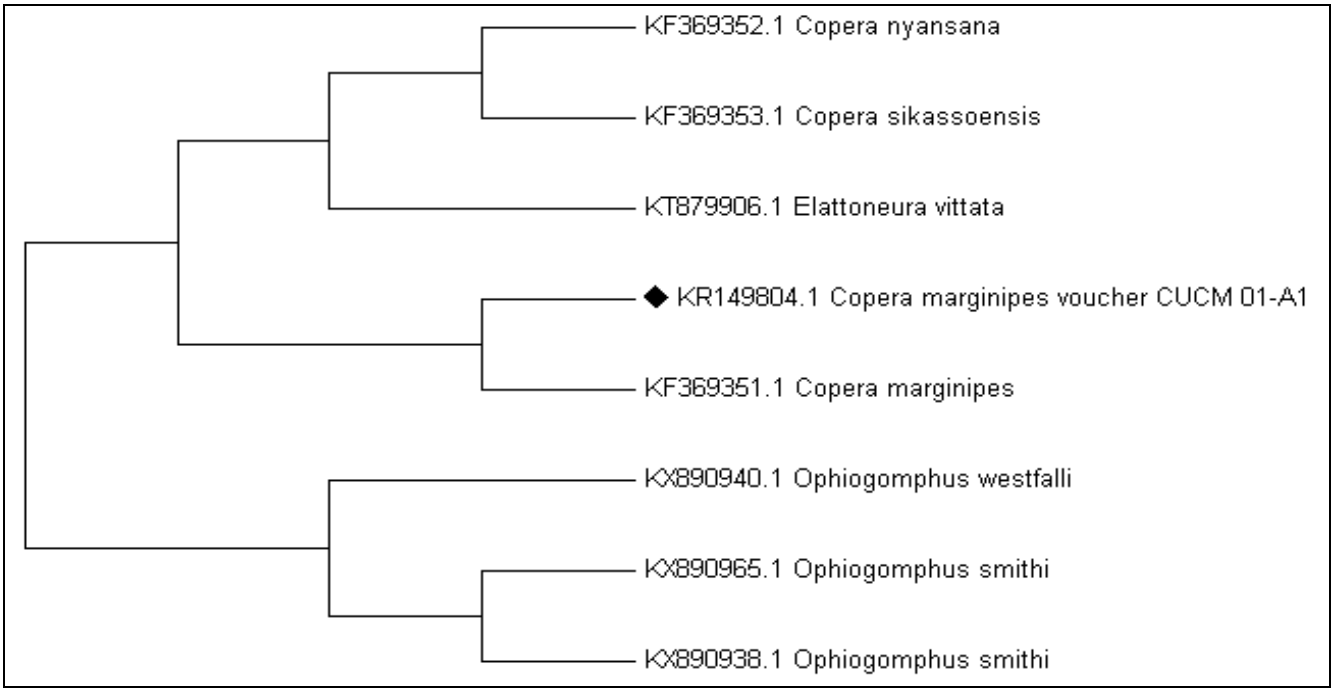

Fig 3: The molecular phylogenetic tree of *Copera marginipes* inferred by NJ tree method

Table 2: The Nucleotide substitution table of *Copera marginipes*

Domain: Data	T(U)	C	A	G	T-1	C-1	A-1	G-1	T-2	C-2	A-2	G-2	T-3	C-3	A-3	G-3
KR149804.1 *Copera marginipes (*KERALA)	34.6	15.8	31.4	18.1	24	15.9	28.4	31.8	43	27.0	13.5	16.5	37	4.5	52.5	6.0
KF369351.1 *Copera marginipes*	34.6	15.8	31.4	18.1	24	15.9	28.4	31.8	43	27.0	13.5	16.5	37	4.5	52.5	6.0
KT879906.1 *Elattoneura vittata*	35.1	15.6	31.9	17.3	25	12.9	29.9	31.8	43	27.0	13.0	17.0	37	7.0	53.0	3.0
KF369352.1 *Copera nyansana*	34.8	16.3	30.3	18.6	23	15.4	28.9	32.3	43	27.0	13.0	17.0	38	6.5	49.0	6.5
KF369353.1 *Copera sikassoensis*	34.4	16.3	31.1	18.1	25	14.4	28.4	32.3	43	27.0	13.0	17.0	36	7.5	52.0	5.0
Avg.	34.8	16.2	31.4	17.6	24	15.2	28.8	32.2	43	27.2	13.3	16.5	38	6.3	52.1	4.1

Conclusion

Copera marginipes is popularly known as 'Yellow bush dart'. The highest diversity of this species has been reported from tropical Asia, Southeastern Asia and New Guinea (Lim *et al*., 2013). Morphological identification of the species was done using taxonomic key (Emiliyamma *et al*., 2005) and with online photographs. The wing venation and other morphological characters of this species clearly described that it is a Plactinimidinae member having the unique features of *Copera marginipes*. Morphological features confirmed a monophyletic assemblage to all Platicnemidae members due to its characteristic feather like tibiae [3]. Most of the male members of this family are having white, yellow, orange, red, blue or black tibiae. Molecular identification and phylogenetic status of this species clearly states that the conserved sequence of cytochrome oxidase I gene doesn't have major evolutionary change as time progresses. Those species reported from Gujarat and Malaysia are 100% sequence similar indicating no means of sympatric speciation. The phylogenetic tree says that the ancestors of Copera genus were splitted at an earlier time with *Copera nyansana* and *Copera sikassoensis* were found in one clade as sister taxa and *Copera marginipes* from Kerala and Malaysia were found in another clade as sister taxa. Phylogenetically its nearest member was found to be *Copera nyansana* and *Copera sikassoensis* with respective divergence of 16.02 and 17.18%. The above result is confirmed by the previous works done by [5]. *Copera marginipes* was found to be evolved for the first time followed by *Copera sikassoensis* and *Copera nyansana*. Thus the result confirmed that all the genera have splitted from one clade indicating monophyletic origin. Most of the morphological unique features indicated phylogeny shown that this genus has been originated in Eastern Asia (Indonesia to Japan) and it is strictly a Palaearctic representative [3]. Thus both morphology and molecular analysis provided a unique result and its molecular taxonomic id can be used to easily spot the species and also to infer evolutionary relationships.

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