

DNA barcoding based on mitochondrial COI gene on some species of family Platycnemididae (Odonata: Zygoptera) from India

Dr. Monika Devi¹ and Dr. Gurinder Kaur Walia^{2*}

¹Assistant Professor, Trinity College Jalandhar, Punjab-144009, India

²Associate Professor, Department of Zoology and Environmental Sciences, Punjabi University, Patiala-147002, Punjab, India

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Abstract

Taxonomically, family Platycnemididae comprises 455 species referable to 43 genera all over the world, while 52 species of 14 genera are present in India. COI gene analysis of 11 species under 6 genera of family Platycnemididae from the states of Himachal Pradesh, Jammu and Kashmir, Kerala, Maharashtra and Meghalaya (India) has been done. The overall data has been used to calculate the sequence divergence at different taxonomic levels in the family Platycnemididae, which shows the hierarchical increase in the K2P values. In the present study, maximum intraspecific distance has been observed as 1.7% in *C. marginipes*, while the only exception is *Coelliccia didyma*, which shows intraspecific distance of 5.2%. Minimum interspecific distance has been observed as 3.4% in *Coelliccia cyaneothorax* and *Coelliccia nemoricola*, while maximum average interspecific distance has been recorded as 18.6±0% in *Calicnemia chaseni* and *Calicnemia eximia*, the only exception with very less distance (0.4±0.1%) in *Coelliccia chromothorax* and *Coelliccia renifera*. Intergeneric divergence has been calculated for all the 6 genera based on K2P distances. The most distant genera have been found to be *Calicnemia* and *Disparoneura* with average K2P distance of 24.2±1.2%, while *Calicnemia* and *Coelliccia* are the closest genera with K2P distance of 17.5±3.5%.

Keywords COI gene; K2P distance; Platycnemididae; Evolutionary affinities; Neighbour-Joining tree.

Significance statement: In the present study, molecular data based on mitochondrial COI gene has been generated for 11 species. Among these, 8 species have been submitted for the first time to NCBI, while 3 species have been submitted for the first time from India

Introduction

India is known for its rich biodiversity of both flora and fauna therefore, integration of molecular techniques, such as DNA barcoding, with traditional taxonomy has been suggested to be a tool to characterize such a huge biodiversity at regional scales. Hebert et al [1] proposed "DNA barcoding" for the documentation and identification of biological diversity based on mitochondrial DNA sequences for the first time. DNA sequences are unique for each species, they can be viewed as genetic 'barcodes' and helpful to solve the problems faced by taxonomists. Odonates possess high level of sexual dimorphism as there are large phenotypic variations among the male and female individuals of the same species, which might be misidentified.

So, DNA barcoding is emerging as an invaluable tool for the authentication of the species.

650 base-pair region of mitochondrial Cytochrome C Oxidase I (COI) gene is being used as the standard barcode for almost all animal groups. Cytochrome oxidase subunit I (COI) gene has two important advantages as a molecular marker. First, the universal primers for this gene are very robust, made recovery of its 5' end from representatives of almost all animal phyla [2], [3]. Second, COI possesses a greater range of phylogenetic signals than any other mitochondrial gene, its third-position nucleotides shows a high incidence of base substitutions, leading to a high rate of molecular evolution that is about three times greater than that of 12S or 16S rDNA [4].

Till date, COI gene sequences for 73 species referable to 33 genera of family Platycnemididae have been submitted, worldwide [5], [6], [7]..... [12]. Moreover, this is the first report on COI gene sequences of 11 species of family Platycnemididae from India and these sequences have been compared with the available data on the congeneric species in the GenBank.

*Corresponding author's ORCID ID: 0000-0003-1130-3261

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Material and Methods

Collection of Specimens and Preservation: Adult specimens of 11 species of Platycnemididae referable to 6 genera were collected from the states of Himachal Pradesh, Meghalaya, Jammu and Kashmir, Kerala, Maharashtra and Meghalaya (India). Some specimens were pinned and stored in fumigated insect boxes for morphological identification, while others were preserved in ethanol on the spot for molecular studies. The specimens were identified by consulting “The Fauna of British India, including Ceylon and Burma” Volume I” [13] and “Dragonflies and Damselflies of Peninsular India-A Field Guide” [14].

DNA extraction, amplification and sequencing: Mitochondrial DNA extraction of 11 species (5 of subfamily Calicnemiinae, 2 of subfamily Platycnemidinae and 4 of subfamily Disparoneurinae) was done from the

samples preserved in alcohol from various localities. The head, legs and thorax were used for the extraction by the technique of kambhampati and Rai [15]. 650 bp region of COI gene was amplified using 1µl each 10µM of primers LCO 1490 and HCO 2198 [2], 12.5 µl of ready to use master mix (containing *Taq* polymerase, 400µM of each dNTPs, 5.5mM of MgCl₂ and reaction buffer) and 1µl of template DNA. The PCR conditions used for amplification were: Initial denaturation at 95°C for 5min, 35 cycles of denaturation at 95°C for 1 min, annealing at 45-50°C for 1 min and elongation at 72°C for 90 sec followed by a final elongation at 72°C for 7 min. The amplified COI gene was run on EtBr stained 1% agarose gel along with 100bp ladder to confirm the length of amplified gene segment. The gene sequences were got sequenced from Yaazh Xenomics, Coimbatore (Tamil Nadu), using Sanger dideoxy method. 30 COI gene sequences for eleven species were submitted to GenBank (Table 1).

Table 1: Details of COI gene sequences of taxa submitted to GenBank

S. No.	Taxa	Place of Collection	Accession No.	Latitude	Longitude
Subfamily- Calicnemiinae					
1.	<i>Calicnemia eximia</i> (Selys, 1863)	Andretta Andretta Chamba Chamba (Himachal Pradesh)	MH183131 MN648199 MN258027 MN378565	32°3'50.04"N 32°3'50.04"N 32°34'12"N 32°34'12"N	76°33'46.8"E 76°33'46.8"E 76°7'48"E 76°7'48"E
2.	<i>Coelliccia chromothorax</i> (Selys, 1891)	Andretta Andretta (Himachal Pradesh)	MN101859 MN365663	32°3'50.04"N 32°3'50.04"N	76°33'46.8"E 76°33'46.8"E
3.	<i>Coelliccia didyma</i> (Selys, 1863)	Nongkhyllem Nongkhyllem (Meghalaya)	MW590680 MW599158	25°34'27.228" N 25°34'27.228" N	91°18'35.5248" E 91°18'35.5248" E
4.	<i>Coelliccia fraseri</i> Laidlaw, 1932	Nongkhyllem Nongkhyllem (Meghalaya)	MK650891 MH183130	25°34'27.228" N 25°34'27.228" N	91°18'35.5248" E 91°18'35.5248" E
5.	<i>Coelliccia renifera</i> (Selys, 1886)	Bilaspur Bilaspur (Himachal Pradesh)	MG518614 MN114981	31°19'48" N 31°19'48" N	76°45'0" E 76°45'0" E
Subfamily-Platycnemidinae					
6.	<i>Copera marginipes</i> (Rambur, 1842)	Mansar lake (Jammu and Kashmir) Andretta Una Bilaspur (Himachal Pradesh)	MG518616 MN201603 MN239486 MN648196	32°41'45.88" N 32°3'50.04"N 31°28'4.8" N 31°19'48" N	75°8'48.5" E 76°33'46.8"E 76°16'15.6"E 76°45'0" E
7.	<i>Copera vittata</i> (Selys, 1863)	Thiruvananthapuram Thiruvananthapuram (Kerala) Nongkhyllem (Meghalaya)	MN442124 MN447532 MN640593	8°29'15.756" N 8°29'15.756" N 25°34'27.228" N	76°55' 26.184"E 76°55' 26.184" E 91°18'35.5248" E
Subfamily- Disparoneurinae					
8.	<i>Disparoneura quadrimaculata</i> (Rambur, 1842)	Nagpur Khandala (Maharashtra)	MN320382 MN648197	21°9'0" N 18°3'28.188" N	79°5' 0" E 74° 1' 34.356" E
9.	<i>Esme cyaneovittata</i> Fraser, 1922	Thiruvananthapuram Kollam Wayanad (Kerala)	MN258026 MN342246 MN395488	8°29'15.756" N 8°52'48" N 11°36'18" N	76°55' 26.184" E 76°36'0" E 76°4'58.8" E
10.	<i>Esme longistyla</i> Fraser, 1931	Wayanad Wayanad Kozhikode (Kerala)	MN417950 MN274522 MN385255	11°36'18" N 11°36'18" N 11°15'0" N	76°4'58.8" E 76°4'58.8" E 75°46'12" E
11.	<i>Prodasineura verticalis</i> (Selys, 1860)	Thiruvananthapuram Kollam Kozhikode (Kerala)	MN304942 MN389528 MN401308	8°29'15.756" N 8°52'48" N 11°15'0" N	76°55' 26.184" E 76°36'0" E 75°46'12" E

Data retrieval from GenBank for sequence alignment and divergence calculation: Corresponding sequences of 14 species belonging to 5 genera of family Platycnemididae were procured directly from the GenBank, deposited by other workers by BLAST (Table 2). All the presently studied species possess distinct barcodes and matched with sequences of congeneric species. All the sequences were aligned, edited and trimmed manually by using ClustalW in MEGA 6 software. Nucleotide divergence within and among species and Intergeneric divergence were calculated by using Kimura 2-parameter (K2P) model of base substitution [16] in Mega 6 software [17].

Table 2: List of COI gene sequences of taxa retrieved from GenBank for alignment

S. No.	Taxa	Country	Accession No.	Authors
1.	<i>Calicnemia chaseni</i> (Laidlaw, 1928)	Malaysia	KF369330	[8]
2.	<i>Calicnemia sinensis</i> Lieftinck, 1984	China	KF369331	[8]
3.	<i>Coelliccia albicauda</i> (Förster in Laidlaw, 1907)	Malaysia	KP978615	[7]
4.	<i>Coelliccia cyanomelas</i> Ris, 1912	China	KP978568	[10]
5.	<i>Coelliccia cyaneothorax</i> Kimmins, 1936	Malaysia	KF369343	[8]
6.	<i>Coelliccia didyma</i> (Selys, 1863)	Malaysia	KP978404	[8]
7.	<i>Coelliccia nemoricola</i> Laidlaw, 1912	Malaysia	KP978588	[10]
8.	<i>Coelliccia octogesima</i> (Selys, 1863)	Malaysia	KP978475	[10]
9.	<i>Coelliccia poungyi</i> Fraser, 1924	Malaysia	KP978529	[10]
10.	<i>Copera marginipes</i> (Rambur, 1842)	Malaysia	KF369351	[7]
11.	<i>Copera nyansana</i> (Förster, 1916)	Malaysia	KF369352	[8]
12.	<i>Copera sikassoensis</i> (Martin, 1912)	Liberia	KF369353	[8]
13.	<i>Esme mudiensis</i> Fraser, 1931	India	KF369386	[8]
14.	<i>Prodasineura sita</i> (Kirby, 1893)	Sri Lanka	KF369512	[8]
15.	<i>Neurobasis longipes</i> Hagen, 1887	Malaysia	KF369461	[8]

Phylogenetic analysis: To derive evolutionary affinities, a total of 45 sequences (30 present + 15 GenBank) were analysed to construct Neighbour-Joining tree using K2P model of substitution with the help of MEGA 6 software [17]. Sequence of *Neurobasis longipes* of the family Calopterygidae was taken as an outgroup.

Results and Discussion

The overall aligned data of 360 bp length shows 199 conserved sites (55.2%), 161 variable sites (44.7%) and 147 parsimony informative sites (40.8%), which represents that COI gene is more conserved. Nucleotide substitution has been calculated by Maximum Composite Likelihood Estimate and the overall transition/transversion bias is found to be $R=0.97$, which indicates transitions to be more prevalent (Table 3). The nucleotide composition in the sequences has been recovered as A=30.4%, T=34.0%, C=19.1% and G=16.5%, which reveals high AT content (64.4%) as earlier reported in the family Platycnemididae [9].

Table 3: Maximum Composite Likelihood Estimate for the Pattern of Nucleotide Substitutions shown by probability of substitution (r) from one base in row to another base in the column out of 100 events

	A	T	C	G
A	-	8.52	4.79	1.23
T	7.61	-	16.71	4.14
C	7.61	29.69	-	4.14
G	2.26	8.52	4.79	-

* labels the sequence of the present study

Intraspecific and Interspecific divergence

Subfamily- Calicnemiinae

Genus *Calicnemia* Strand, 1928

Intraspecific divergence: This is the only species of the genus. Four specimens of *Calicnemia eximia* have been collected from Andretta and Chamba (H. P.) and possesses 0% intraspecific divergence (Table 4).

Table 4: The intraspecific divergence of sequences

S. No.	Species (Accession no.)	Species (Accession no.)	Divergence (%)
Subfamily- Calicnemiinae			
Genus <i>Calicnemia</i> Strand, 1928			
1.	<i>Calicnemia eximia</i> MN258027*	<i>Calicnemia eximia</i> MN648199*	0
2.	<i>Calicnemia eximia</i> MN258027*	<i>Calicnemia eximia</i> MH183131*	0
3.	<i>Calicnemia eximia</i> MN258027*	<i>Calicnemia eximia</i> MN378565*	0
4.	<i>Calicnemia eximia</i> MN378565*	<i>Calicnemia eximia</i> MH183131*	0
5.	<i>Calicnemia eximia</i> MN378565*	<i>Calicnemia eximia</i> MN648199*	0
6.	<i>Calicnemia eximia</i> MH183131*	<i>Calicnemia eximia</i> MN648199*	0
Genus <i>Coelliccia</i> Kirby, 1890			
1	<i>Coelliccia chromothorax</i> MN101859*	<i>Coelliccia chromothorax</i> MN365663*	0
2	<i>Coelliccia didyma</i> MW599158*	<i>Coelliccia didyma</i> MW590680*	0

3	<i>Coeliccia didyma</i> KP978404	<i>Coeliccia didyma</i> MW599158*	5.2
4	<i>Coeliccia didyma</i> KP978404	<i>Coeliccia didyma</i> MW590680*	5.2
5	<i>Coeliccia fraseri</i> MH183130*	<i>Coeliccia fraseri</i> MK650891*	0
6	<i>Coeliccia renifera</i> MN114981*	<i>Coeliccia renifera</i> MG518614*	0.2
Subfamily- Platycnemidinae			
Genus Copera Kirby, 1890			
1	<i>Copera marginipes</i> MN239486*	<i>Copera marginipes</i> MN648196*	0
2	<i>Copera marginipes</i> MN239486*	<i>Copera marginipes</i> MN201603*	0
3	<i>Copera marginipes</i> MN648196*	<i>Copera marginipes</i> MN201603*	0
4	<i>Copera marginipes</i> MN239486*	<i>Copera marginipes</i> KF369351	1.4
5	<i>Copera marginipes</i> MN648196*	<i>Copera marginipes</i> KF369351	1.4
6	<i>Copera marginipes</i> MN201603*	<i>Copera marginipes</i> KF369351	1.4
7	<i>Copera marginipes</i> MN239486*	<i>Copera marginipes</i> MG518616*	0.3
8	<i>Copera marginipes</i> MN648196*	<i>Copera marginipes</i> MG518616*	0.3
9	<i>Copera marginipes</i> MN201603*	<i>Copera marginipes</i> MG518616*	0.3
10	<i>Copera marginipes</i> KF369351	<i>Copera marginipes</i> MG518616*	1.7
11	<i>Copera vittata</i> MN640593*	<i>Copera vittata</i> MN447532*	0
12	<i>Copera vittata</i> MN640593*	<i>Copera vittata</i> MN442124*	0
13	<i>Copera vittata</i> MN447532*	<i>Copera vittata</i> MN442124*	0
Subfamily- Disparoneurinae			
Genus Disparoneura Selys, 1860			
1.	<i>Disparoneura qudrimaculata</i> MN320382*	<i>Disparoneura qudrimaculata</i> MN648197*	0
Genus Esme Fraser, 1922			
1	<i>Esme cyaneovittata</i> MN395488*	<i>Esme cyaneovittata</i> MN342246*	0
2	<i>Esme cyaneovittata</i> MN395488*	<i>Esme cyaneovittata</i> MN258026*	0
3	<i>Esme cyaneovittata</i> MN342246*	<i>Esme cyaneovittata</i> MN258026*	0
4	<i>Esme longistyla</i> MN385255*	<i>Esme longistyla</i> MN274522*	0
5	<i>Esme longistyla</i> MN385255*	<i>Esme longistyla</i> MN417950*	0.3
6	<i>Esme longistyla</i> MN274522*	<i>Esme longistyla</i> MN417950*	0.3
Prodasineura Cowley, 1934			
1	<i>Prodasineura verticalis</i> MN401308	<i>Prodasineura verticalis</i> MN389528*	0
2	<i>Prodasineura verticalis</i> MN401308	<i>Prodasineura verticalis</i> MN304942*	0
3	<i>Prodasineura verticalis</i> MN389528	<i>Prodasineura verticalis</i> MN304942*	0

*indicates specimens of the present study

Interspecific divergence: Interspecific distances have been calculated for 6 COI sequences of 3 species of genus

Calicnemia, which includes 4 sequences of *Calicnemia eximia* (present study), 1 sequence each of *Calicnemia chaseni* and *Calicnemia sinensis* (from the GenBank). All the species show quite high average interspecific divergence as 16.6±0% for *Calicnemia chaseni* and *Calicnemia sinensis*, 17.9±0% for *Calicnemia sinensis* and *Calicnemia eximia* and 18.6±0% for *Calicnemia chaseni* and *Calicnemia eximia* (Table 5).

Table 5: The interspecific divergence of species with congeners

S. No.	Species (Accession no.)	Species (Accession no.)	Divergence (%)	Average Divergence (%)
Subfamily- Calicnemiinae				
Genus Calicnemia Strand, 1928				
1.	<i>Calicnemia chaseni</i> KF369330	<i>Calicnemia sinensis</i> KF369331	16.6	-
2.	<i>Calicnemia chaseni</i> KF369330	<i>Calicnemia eximia</i> MH183131*	18.6	18.6±0
3.	<i>Calicnemia chaseni</i> KF369330	<i>Calicnemia eximia</i> MN648199*	18.6	
4.	<i>Calicnemia chaseni</i> KF369330	<i>Calicnemia eximia</i> MN378565*	18.6	
5.	<i>Calicnemia chaseni</i> KF369330	<i>Calicnemia eximia</i> MN258027*	18.6	
6.	<i>Calicnemia sinensis</i> KF369331	<i>Calicnemia eximia</i> MH183131*	17.9	17.9±0
7.	<i>Calicnemia sinensis</i> KF369331	<i>Calicnemia eximia</i> MN378565*	17.9	
8.	<i>Calicnemia sinensis</i> KF369331	<i>Calicnemia eximia</i> MN258027*	17.9	
9.	<i>Calicnemia sinensis</i> KF369331	<i>Calicnemia eximia</i> MN648199*	17.9	
Genus Coeliccia Kirby, 1890				
1.	<i>Coeliccia albicauda</i> KP978615	<i>Coeliccia cyanomelas</i> KP978568	14.4	-
2.	<i>Coeliccia albicauda</i> KP978615	<i>Coeliccia cyaneothorax</i> KF369343	10.4	-
3.	<i>Coeliccia albicauda</i> KP978615	<i>Coeliccia nemoricola</i> KP978588	11.4	-
4.	<i>Coeliccia albicauda</i> KP978615	<i>Coeliccia poungyi</i> KP978529	12.1	-
5.	<i>Coeliccia albicauda</i> KP978615	<i>Coeliccia octogesima</i> KP978475	11.7	-
6.	<i>Coeliccia albicauda</i> KP978615	<i>Coeliccia didyma</i> KP978404	13.1	14.9±1.5
7.	<i>Coeliccia albicauda</i> KP978615	<i>Coeliccia didyma</i> MW599158*	15.8	
8.	<i>Coeliccia albicauda</i>	<i>Coeliccia didyma</i>	15.8	

	KP978615	MW590680*		
9.	<i>Coelliccia albicauda</i> KP978615	<i>Coelliccia chromothorax</i> MN101859*	12.1	12.1±0
10.	<i>Coelliccia albicauda</i> KP978615	<i>Coelliccia chromothorax</i> MN365663*	12.1	
11.	<i>Coelliccia albicauda</i> KP978615	<i>Coelliccia fraseri</i> MH183130*	11.1	11.1±0
12.	<i>Coelliccia albicauda</i> KP978615	<i>Coelliccia fraseri</i> MK650891*	11.1	
13.	<i>Coelliccia albicauda</i> KP978615	<i>Coelliccia renifera</i> MN114981*	12.4	12.2±0.2
14.	<i>Coelliccia albicauda</i> KP978615	<i>Coelliccia renifera</i> MG518614*	12.1	
15.	<i>Coelliccia chromothorax</i> MN101859*	<i>Coelliccia fraseri</i> MH183130*	13.5	13.5±0
16.	<i>Coelliccia chromothorax</i> MN101859*	<i>Coelliccia fraseri</i> MK650891*	13.5	
17.	<i>Coelliccia chromothorax</i> MN365663*	<i>Coelliccia fraseri</i> MH183130*	13.5	
18.	<i>Coelliccia chromothorax</i> MN365663*	<i>Coelliccia fraseri</i> MK650891*	13.5	
19.	<i>Coelliccia chromothorax</i> MN101859*	<i>Coelliccia renifera</i> MN114981*	0.6	0.4±0.1
20.	<i>Coelliccia chromothorax</i> MN365663*	<i>Coelliccia renifera</i> MN114981*	0.6	
21.	<i>Coelliccia chromothorax</i> MN101859*	<i>Coelliccia renifera</i> MG518614*	0.3	
22.	<i>Coelliccia chromothorax</i> MN365663*	<i>Coelliccia renifera</i> MG518614*	0.3	
23.	<i>Coelliccia cyaneothorax</i> KF369343	<i>Coelliccia nemoricola</i> KP978588	3.4	-
24.	<i>Coelliccia cyaneothorax</i> KF369343	<i>Coelliccia poungyi</i> KP978529	13.7	-
25.	<i>Coelliccia cyaneothorax</i> KF369343	<i>Coelliccia octogesima</i> KP978475	8.6	-
26.	<i>Coelliccia cyaneothorax</i> KF369343	<i>Coelliccia didyma</i> KP978404	14.8	16.2±1.2
27.	<i>Coelliccia cyaneothorax</i> KF369343	<i>Coelliccia didyma</i> MW599158*	16.9	
28.	<i>Coelliccia cyaneothorax</i> KF369343	<i>Coelliccia didyma</i> MW590680*	16.9	
29.	<i>Coelliccia cyaneothorax</i> KF369343	<i>Coelliccia chromothorax</i> MN101859*	15.2	15.2±0
30.	<i>Coelliccia cyaneothorax</i> KF369343	<i>Coelliccia chromothorax</i> MN365663*	15.2	
31.	<i>Coelliccia cyaneothorax</i> KF369343	<i>Coelliccia fraseri</i> MH183130*	13.4	13.4±0
32.	<i>Coelliccia</i>	<i>Coelliccia</i>	13.4	

	<i>cyaneothorax</i> KF369343	<i>fraseri</i> MK650891*		
33.	<i>Coelliccia cyaneothorax</i> KF369343	<i>Coelliccia renifera</i> MN114981*	15.6	15.4±0.2
34.	<i>Coelliccia cyaneothorax</i> KF369343	<i>Coelliccia renifera</i> MG518614*	15.2	
35.	<i>Coelliccia cyanomelas</i> KP978568	<i>Coelliccia cyaneothorax</i> KF369343	12.7	-
36.	<i>Coelliccia cyanomelas</i> KP978568	<i>Coelliccia nemoricola</i> KP978588	11.7	-
37.	<i>Coelliccia cyanomelas</i> KP978568	<i>Coelliccia poungyi</i> KP978529	15.1	-
38.	<i>Coelliccia cyanomelas</i> KP978568	<i>Coelliccia octogesima</i> KP978475	12.4	-
39.	<i>Coelliccia cyanomelas</i> KP978568	<i>Coelliccia didyma</i> KP978404	14.9	15.8±0.8
40.	<i>Coelliccia cyanomelas</i> KP978568	<i>Coelliccia didyma</i> MW599158*	16.3	
41.	<i>Coelliccia cyanomelas</i> KP978568	<i>Coelliccia didyma</i> MW590680*	16.3	
42.	<i>Coelliccia cyanomelas</i> KP978568	<i>Coelliccia chromothorax</i> MN101859*	13.9	13.9±0
43.	<i>Coelliccia cyanomelas</i> KP978568	<i>Coelliccia chromothorax</i> MN365663*	13.9	
44.	<i>Coelliccia cyanomelas</i> KP978568	<i>Coelliccia fraseri</i> MH183130*	16.2	16.2±0
45.	<i>Coelliccia cyanomelas</i> KP978568	<i>Coelliccia fraseri</i> MK650891*	16.2	
46.	<i>Coelliccia cyanomelas</i> KP978568	<i>Coelliccia renifera</i> MN114981*	14.2	14.0±0.2
47.	<i>Coelliccia cyanomelas</i> KP978568	<i>Coelliccia renifera</i> MG518614*	13.9	
48.	<i>Coelliccia didyma</i> MW599158*	<i>Coelliccia chromothorax</i> MN101859*	16.4	15.3±1.2
49.	<i>Coelliccia didyma</i> MW599158*	<i>Coelliccia chromothorax</i> MN365663*	16.4	
50.	<i>Coelliccia didyma</i> KP978404	<i>Coelliccia chromothorax</i> MN101859*	13.9	
51.	<i>Coelliccia didyma</i> KP978404	<i>Coelliccia chromothorax</i> MN365663*	13.9	14.7±0.6
52.	<i>Coelliccia didyma</i> MW590680*	<i>Coelliccia chromothorax</i> MN101859*	15.3	
53.	<i>Coelliccia didyma</i> MW590680*	<i>Coelliccia chromothorax</i> MN365663*	16.4	
54.	<i>Coelliccia didyma</i> MW599158*	<i>Coelliccia fraseri</i> MH183130*	15.2	14.7±0.6
55.	<i>Coelliccia didyma</i> MW599158*	<i>Coelliccia fraseri</i> MK650891*	15.2	

56.	<i>Coelliccia didyma</i> MW590680*	<i>Coelliccia fraseri</i> MH183130*	15.2	15.6±1.2
57.	<i>Coelliccia didyma</i> MW590680*	<i>Coelliccia fraseri</i> MK650891*	15.2	
58.	<i>Coelliccia didyma</i> KP978404	<i>Coelliccia fraseri</i> MH183130*	13.9	
59.	<i>Coelliccia didyma</i> KP978404	<i>Coelliccia fraseri</i> MK650891*	13.9	
60.	<i>Coelliccia didyma</i> MW599158*	<i>Coelliccia renifera</i> MN114981*	16.7	
61.	<i>Coelliccia didyma</i> MW599158*	<i>Coelliccia renifera</i> MG518614*	16.3	
62.	<i>Coelliccia didyma</i> KP978404	<i>Coelliccia renifera</i> MN114981*	14.2	
63.	<i>Coelliccia didyma</i> KP978404	<i>Coelliccia renifera</i> MG518614*	13.9	
64.	<i>Coelliccia didyma</i> MW590680*	<i>Coelliccia renifera</i> MN114981*	16.7	
65.	<i>Coelliccia didyma</i> MW590680*	<i>Coelliccia renifera</i> MG518614*	16.3	
66.	<i>Coelliccia fraseri</i> MH183130*	<i>Coelliccia renifera</i> MN114981*	13.9	13.7±0.2
67.	<i>Coelliccia fraseri</i> MH183130*	<i>Coelliccia renifera</i> MG518614*	13.5	
68.	<i>Coelliccia fraseri</i> MK650891*	<i>Coelliccia renifera</i> MN114981*	13.9	
69.	<i>Coelliccia fraseri</i> MK650891*	<i>Coelliccia renifera</i> MG518614*	13.5	
70.	<i>Coelliccia nemoricola</i> KP978588	<i>Coelliccia poungyi</i> KP978529	13.1	
71.	<i>Coelliccia nemoricola</i> KP978588	<i>Coelliccia octogesima</i> KP978475	7.4	-
72.	<i>Coelliccia nemoricola</i> KP978588	<i>Coelliccia didyma</i> KP978404	15.1	16.5±1.2
73.	<i>Coelliccia nemoricola</i> KP978588	<i>Coelliccia didyma</i> MW599158*	17.2	
74.	<i>Coelliccia nemoricola</i> KP978588	<i>Coelliccia didyma</i> MW590680*	17.2	
75.	<i>Coelliccia nemoricola</i> KP978588	<i>Coelliccia chromothorax</i> MN101859*	14.8	
76.	<i>Coelliccia nemoricola</i> KP978588	<i>Coelliccia chromothorax</i> MN365663*	14.8	14.8±0
77.	<i>Coelliccia nemoricola</i> KP978588	<i>Coelliccia fraseri</i> MH183130*	13.4	13.4±0
78.	<i>Coelliccia nemoricola</i> KP978588	<i>Coelliccia fraseri</i> MK650891*	13.4	
79.	<i>Coelliccia nemoricola</i>	<i>Coelliccia renifera</i>	15.2	15±0.2

80.	KP978588 <i>Coelliccia nemoricola</i> KP978588	MN114981* <i>Coelliccia renifera</i> MG518614*	14.8	16.8±0.5	
81.	<i>Coelliccia octogesima</i> KP978475	<i>Coelliccia didyma</i> KP978404	16.2		
82.	<i>Coelliccia octogesima</i> KP978475	<i>Coelliccia didyma</i> MW599158*	17.2		
83.	<i>Coelliccia octogesima</i> KP978475	<i>Coelliccia didyma</i> MW590680*	17.2		
84.	<i>Coelliccia octogesima</i> KP978475	<i>Coelliccia chromothorax</i> MN101859*	14.9		14.9±0
85.	<i>Coelliccia octogesima</i> KP978475	<i>Coelliccia chromothorax</i> MN365663*	14.9		
86.	<i>Coelliccia octogesima</i> KP978475	<i>Coelliccia fraseri</i> MH183130*	12.4		12.4±0
87.	<i>Coelliccia octogesima</i> KP978475	<i>Coelliccia fraseri</i> MK650891*	12.4		
88.	<i>Coelliccia octogesima</i> KP978475	<i>Coelliccia renifera</i> MN114981*	15.2		15±0.2
89.	<i>Coelliccia octogesima</i> KP978475	<i>Coelliccia renifera</i> MG518614*	14.8		
90.	<i>Coelliccia poungyi</i> KP978529	<i>Coelliccia octogesima</i> KP978475	12.8	12.8±0	
91.	<i>Coelliccia poungyi</i> KP978529	<i>Coelliccia didyma</i> KP978404	16.2	17.4±0	
92.	<i>Coelliccia poungyi</i> KP978529	<i>Coelliccia didyma</i> MW599158*	17.4		
93.	<i>Coelliccia poungyi</i> KP978529	<i>Coelliccia didyma</i> MW590680*	17.4		
94.	<i>Coelliccia poungyi</i> KP978529	<i>Coelliccia chromothorax</i> MN101859*	16.0	16.0±0	
95.	<i>Coelliccia poungyi</i> KP978529	<i>Coelliccia chromothorax</i> MN365663*	16.0		
96.	<i>Coelliccia poungyi</i> KP978529	<i>Coelliccia fraseri</i> MH183130*	15.1	15.1±0	
97.	<i>Coelliccia poungyi</i> KP978529	<i>Coelliccia fraseri</i> MK650891*	15.1		
98.	<i>Coelliccia poungyi</i> KP978529	<i>Coelliccia renifera</i> MN114981*	16.3	16.1±0.2	
99.	<i>Coelliccia poungyi</i> KP978529	<i>Coelliccia renifera</i> MG518614*	16.0		
Subfamily- Platycnemidinae					
Genus <i>Copera</i> Kirby, 1890					
1.	<i>Copera marginipes</i> MN239486*	<i>Copera nyansana</i> KF369352	15.9	15.5±0.6	
2.	<i>Copera marginipes</i> MN648196*	<i>Copera nyansana</i> KF369352	15.9		
3.	<i>Copera marginipes</i>	<i>Copera nyansana</i>	15.9		

	MN201603*	KF369352		
4.	<i>Copera marginipes</i> KF369351	<i>Copera nyansana</i> KF369352	14.4	
5.	<i>Copera marginipes</i> MG518616*	<i>Copera nyansana</i> KF369352	15.5	
6.	<i>Copera marginipes</i> MN239486*	<i>Copera sikassoensis</i> KF369353	16.5	16.3±0.1
7.	<i>Copera marginipes</i> MN648196*	<i>Copera sikassoensis</i> KF369353	16.5	
8.	<i>Copera marginipes</i> MN201603*	<i>Copera sikassoensis</i> KF369353	16.5	
9.	<i>Copera marginipes</i> KF369351	<i>Copera sikassoensis</i> KF369353	16.2	
10.	<i>Copera marginipes</i> MG518616*	<i>Copera sikassoensis</i> KF369353	16.2	
11.	<i>Copera nyansana</i> KF369352	<i>Copera sikassoensis</i> KF369353	12.9	-
12.	<i>Copera marginipes</i> MN239486*	<i>Copera vittata</i> MN640593*	14.8	14.6±0.3
13.	<i>Copera marginipes</i> MN648196*	<i>Copera vittata</i> MN640593*	14.8	
14.	<i>Copera marginipes</i> MN201603*	<i>Copera vittata</i> MN640593*	14.8	
15.	<i>Copera marginipes</i> KF369351	<i>Copera vittata</i> MN640593*	14.1	
16.	<i>Copera marginipes</i> MG518616*	<i>Copera vittata</i> MN640593*	14.5	
17.	<i>Copera marginipes</i> MN239486*	<i>Copera vittata</i> MN447532*	14.8	
18.	<i>Copera marginipes</i> MN648196*	<i>Copera vittata</i> MN447532*	14.8	
19.	<i>Copera marginipes</i> MN201603*	<i>Copera vittata</i> MN447532*	14.8	
20.	<i>Copera marginipes</i> KF369351	<i>Copera vittata</i> MN447532*	14.1	
21.	<i>Copera marginipes</i> MG518616*	<i>Copera vittata</i> MN447532*	14.5	
22.	<i>Copera marginipes</i> MN239486*	<i>Copera vittata</i> MN442124*	14.8	
23.	<i>Copera marginipes</i> MN648196*	<i>Copera vittata</i> MN442124*	14.8	
24.	<i>Copera marginipes</i> MN201603*	<i>Copera vittata</i> MN442124*	14.8	
25.	<i>Copera marginipes</i> KF369351	<i>Copera vittata</i> MN442124*	14.1	
26.	<i>Copera marginipes</i> MG518616*	<i>Copera vittata</i> MN442124*	14.5	
27.	<i>Copera</i>	<i>Copera</i>	14.1	

	<i>nyansana</i> KF369352	<i>vittata</i> MN640593*		14.1±0
28.	<i>Copera nyansana</i> KF369352	<i>Copera vittata</i> MN442124*	14.1	
29.	<i>Copera nyansana</i> KF369352	<i>Copera vittata</i> MN447532*	14.1	
30.	<i>Copera sikassoensis</i> KF369353	<i>Copera vittata</i> MN442124*	12.7	12.7±0
31.	<i>Copera sikassoensis</i> KF369353	<i>Copera vittata</i> MN640593*	12.7	
32.	<i>Copera sikassoensis</i> KF369353	<i>Copera vittata</i> MN447532*	12.7	
Genus <i>Esme</i> Fraser, 1922				
1.	<i>Esme cyaneovittata</i> MN395488*	<i>Esme longistyla</i> MN385255*	15.0	15±0
2.	<i>Esme cyaneovittata</i> MN342246*	<i>Esme longistyla</i> MN385255*	15.0	
3.	<i>Esme cyaneovittata</i> MN258026*	<i>Esme longistyla</i> MN385255*	15.0	
4.	<i>Esme cyaneovittata</i> MN395488*	<i>Esme longistyla</i> MN274522*	15.0	
5.	<i>Esme cyaneovittata</i> MN342246*	<i>Esme longistyla</i> MN274522*	15.0	
6.	<i>Esme cyaneovittata</i> MN258026*	<i>Esme longistyla</i> MN274522*	15.0	
7.	<i>Esme cyaneovittata</i> MN395488*	<i>Esme longistyla</i> MN417950*	15.0	
8.	<i>Esme cyaneovittata</i> MN342246*	<i>Esme longistyla</i> MN417950*	15.0	
9.	<i>Esme cyaneovittata</i> MN258026*	<i>Esme longistyla</i> MN417950*	15.0	
10.	<i>Esme cyaneovittata</i> MN395488*	<i>Esme mudiensis</i> KF369386	11.6	
11.	<i>Esme cyaneovittata</i> MN342246*	<i>Esme mudiensis</i> KF369386	11.6	
12.	<i>Esme cyaneovittata</i> MN258026*	<i>Esme mudiensis</i> KF369386	11.6	
13.	<i>Esme longistyla</i> MN385255*	<i>Esme mudiensis</i> KF369386	17.4	
14.	<i>Esme longistyla</i> MN274522*	<i>Esme mudiensis</i> KF369386	17.4	
15.	<i>Esme longistyla</i> MN417950*	<i>Esme mudiensis</i> KF369386	17.6	
<i>Prodasineura</i> Cowley, 1934				
1	<i>Prodasineura sita</i> KF369512	<i>Prodasineura verticalis</i> MN401308*	17.2	17.2±0
2	<i>Prodasineura sita</i> KF369512	<i>Prodasineura verticalis</i> MN389528*	17.2	
3	<i>Prodasineura</i>	<i>Prodasineura</i>	17.2	

	<i>sita</i> KF369512	<i>verticalis</i> MN304942*		
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*indicates specimens of the present study

Genus *Coellicia* Kirby, 1890

Intraspecific divergence: Intraspecific divergences have been calculated for four species of genus *Coellicia*. It is 0% in two specimens of *Coellicia chromothorax* (Andretta, H. P.), two specimens of *Coellicia fraseri* (Nongkhylllem, Meghalaya), two specimens of *Coellicia renifera* (Bilaspur, H. P.) and two specimens of *Coellicia didyma* (Nongkhylllem, Meghalaya). Moreover, sequences of *Coellicia didyma* show high intraspecific divergence 5.2% with the sequences of same species from Malaysia [8], which is due to geographical isolation of the species (Table 4).

Interspecific divergence: To analyse the interspecific distance, 14 sequences (8 present + 7 GenBank) of genus *Coellicia* have been compared. These are 1 sequence each of *Coellicia albicauda*, *Coellicia cyaneothorax*, *Coellicia cyanomelas*, *Coellicia didyma*, *Coellicia octogesima*, *Coellicia nemoricola* and *Coellicia poungyi* retrieved from the GenBank and 2 sequences each of *Coellicia chromothorax*, *Coellicia didyma*, *Coellicia fraseri* and *Coellicia renifera* of the present study. Minimum average interspecific distance is found to be (0.4±0.1%) in *Coellicia chromothorax* and *Coellicia renifera*, while maximum interspecific distance (17.4±0%) has been observed in *Coellicia poungyi* and *Coellicia didyma* (Table 5).

Subfamily- Platycnemidinae

Genus *Copera* Kirby, 1890

Intraspecific divergence: 4 COI gene sequences of *C. marginipes* show 0-0.3% intraspecific divergence from (Mansar lake, Jammu and Kashmir) and (Andretta, Una, Bilaspur, H. P.) and it is 1.4-1.7% in the sequences of same species from Malaysia [7], which is due to geographical isolation of the species. Three sequences of *Copera vittata* from (Nongkhylllem, Meghalaya) and (Thiruvananthapuram, Kerala) show 0% intraspecific distance (Table 4).

Interspecific divergence: 10 COI sequences (7 present + 3 GenBank) have been used to calculate interspecific divergence. These are 4 of *Copera marginipes* and 3 of *Copera vittata* of the present study, while 1 sequence each of *Copera marginipes*, *Copera nyansana* and *Copera sikassoensis* procured from the GenBank. Minimum average interspecific distance is (12.7±0%) in *Copera vittata* and *Copera sikassoensis*, while maximum interspecific distance is (16.3±0.1%) in *Copera marginipes* and *Copera sikassoensis* (Table 5).

Subfamily- Disparoneurinae

Genus *Disparoneura* Selys, 1860

Intraspecific divergence: Two specimens of *Disparoneura quadrimaculata* collected from Nagpur, Khandala (Maharashtra) show 0% intraspecific divergence (Table 4).

Interspecific divergence: Interspecific divergence for genus *Disparoneura* can not be calculated because no data is available in the GenBank.

Genus *Esme* Fraser, 1922

Intraspecific divergence: 3 sequences of *Esme cyaneovittata* from (Thiruvananthapuram, Kollam and Wayanad, Kerala) show 0% divergence, while in three sequences of *Esme longistyla* from (Wayanad and Kozhikode, Kerala) ranges from 0 to 0.3% (Table 4).

Interspecific divergence: Interspecific distances among three species, *Esme cyaneovittata*, *Esme longistyla* and *Esme mudiensis* have been calculated. In total, 7 sequences including 1 sequence of *Esme mudiensis* (retrieved from GenBank) and 3 sequence each of *Esme cyaneovittata* and *Esme longistyla* (present study). Average interspecific distance is (11.6±0%) for *Esme cyaneovittata* and *Esme mudiensis*, (15±0%) for *Esme cyaneovittata* and *Esme longistyla* and (17.4±0.1%) for *Esme mudiensis* and *Esme longistyla* (Table 5).

Prodasineura Cowley, 1934

Intraspecific divergence: Three specimens of *Prodasineura verticalis* from (Thiruvananthapuram, Kollam and Kozhikode, Kerala) show 0% sequence divergence (Table 4).

Interspecific divergence: Interspecific divergence has been calculated for *Prodasineura verticalis* (3 sequences) of the present study and *Prodasineura sita* (1 sequence) from Sri Lanka as 17.2±0% (Table 5).

The overall data analysed for sequence divergence at different taxonomic levels in the family Platycnemididae shows the hierarchical increase in the K2P values. In the present study, maximum intraspecific distance has been observed as 1.7% in *C. marginipes*, which is in accordance to the earlier studied species of family Platycnemididae as Lim et al [7] observed 0-1.08% in three species of genus *Copera*: *Copera ciliata* (now *Pseudocopera ciliata*), *Copera marginipes* and *Copera vittata* from Malaysia and Bergmann et al [6] found 0-0.2% in 8 individuals of *Chlorocnemis abbotti* (now *Allocnemis abbotti*) from Europe and Africa. The only exception is *Coellicia didyma* which shows high intraspecific distance of 5.2% during the present study.

Minimum interspecific distance has been observed as 3.4% in *Coellicia cyaneothorax* and *Coellicia nemoricola*, while maximum average interspecific distance is found as 18.6±0% in *Calicnemia chaseni* and *Calicnemia eximia* as earlier observed in other species of family Platycnemididae as 9.41% to 12.82% in three species of genus *Copera*: *Copera ciliata* (now *Pseudocopera ciliata*),

Copera marginipes and *Copera vittata* from Malaysia by Lim et al [7]. The only exception is (0.4±0.1%) very less distance in *Coelliccia chromothorax* and *Coelliccia renifera*.

Intergeneric divergence: Intergeneric divergence has been calculated for all the 6 genera based on K2P distances. The most distant genera are found to be *Calicnemia* and *Disparoneura* with average K2P distance of 24.2±1.2%, while *Calicnemia* and *Coelliccia* are the closest genera with K2P distance of 17.5±3.5% (Table 6). These results are in accordance to the earlier reports of the family Platycnemididae as intergeneric divergence of 13.90 to 15.39% in *Copera* and *Coelliccia*, 13.66 to 14.79% in *Copera* and *Prodasineura* and 14.43 to 14.46% in *Coelliccia* and *Prodasineura* by Lim et al [7].

Table 6: Average intergeneric divergence (%) among different genera

	<i>Calicnemia</i>	<i>Coelliccia</i>	<i>Copera</i>	<i>Disparoneura</i>	<i>Esme</i>	<i>Prodasineura</i>
<i>Calicnemia</i>						
<i>Coelliccia</i>	17.5±3.5					
<i>Copera</i>	18.6±1.3	18.9±1.9				
<i>Disparoneura</i>	24.2±1.2	23.4±1.3	23.3±1.0			
<i>Esme</i>	22.2±1.3	20.1±1.8	20.6±0.7	23.5±0.6		
<i>Prodasineura</i>	22.4±2.1	20.9±1.1	20.1±1.7	21.3±0.5	22.5±1.9	

Phylogenetic analysis and evolutionary relationships: An attempt has been made to study evolutionary relationships of the Platycnemid species by constructing NJ tree based on present sequences (30) and sequences (15) retrieved from GenBank (Fig. 1). Phylogenetic tree based on NJ method divides the family Platycnemididae into two clusters, cluster one composed of subfamily Disparoneurinae containing 3 species of genus *Esme* (*Esme longistyla*, *Esme mudiensis* and *Esme cyaneovittata*) and the second group further splits into two subgroups with one composed of subfamily Disparoneurinae and the

other consist of subfamilies Platycnemidinae and Calicnemiinae, which show sister group relationships. Within subfamily Disparoneurinae, two clusters are formed, one containing *Disparoneura quadrimaculata* and the other containing *Prodasineura verticalis* and *Prodasineura sita*.

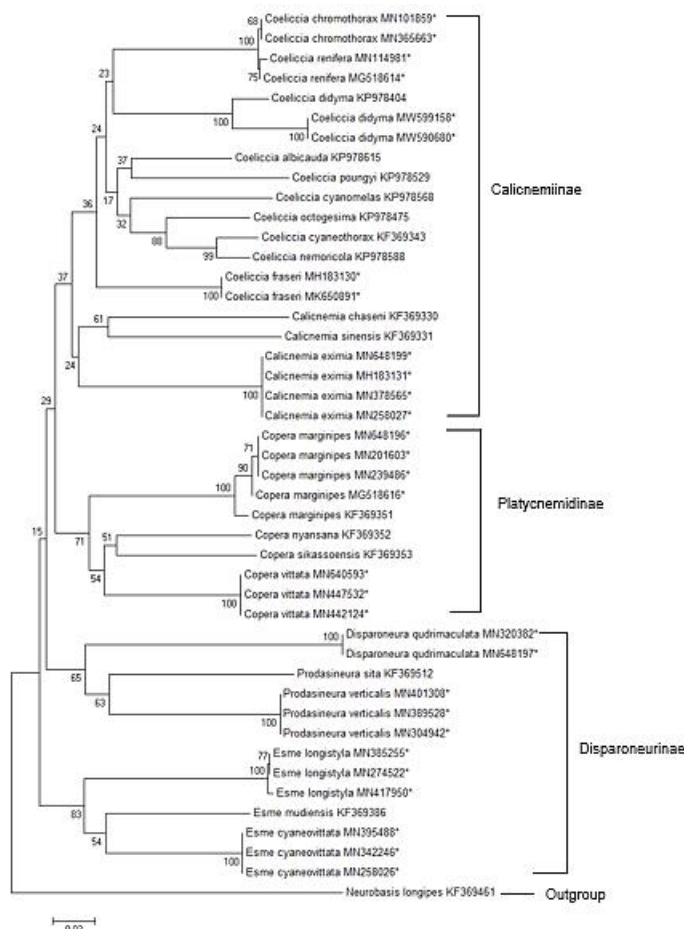


Fig. 1 Molecular Phylogenetic analysis by Neighbor Joining method

Subfamily Platycnemidinae divides into two clusters, one containing *Copera marginipes* and other containing *Copera nyansana*, *Copera sikassoensis* and *Copera vittata*. Subfamily Calicnemiinae gives rise to two clusters with one containing 3 species of genus *Calicnemia* (*Calicnemia chaseni*, *Calicnemia sinensis* and *Calicnemia eximia*) and the other containing 10 species of genus *Coelliccia* (*Coelliccia chromothorax*, *Coelliccia renifera*, *Coelliccia didyma*, *Coelliccia albicauda*, *Coelliccia poungyi*, *Coelliccia cyanomelas*, *Coelliccia octogesima*, *Coelliccia cyaneothorax*, *Coelliccia nemoricola*, *Coelliccia fraseri*).

Phylogenetic analysis depicts that subfamily Disparoneurinae appear to be more closely related to the Platycnemidinae than to Calicnemiinae. Earlier, subfamily Disparoneurinae (Old World ‘Protoneurids’) was present under the family Protoneuridae (New World ‘Protoneurids’) but currently it is placed under the family Platycnemididae based on the phylogenetic analysis of nuclear genes (18S rRNA, 28S rRNA, EF-1α, H3) and

mitochondrial genes (COI, COII, 12S rRNA, 16S rRNA) [7], [8], [18], [19], [20]. In subfamily Calicnemiinae, species of genus *Calicnemia* show sister group relationships with species of genus *Coeliccia* as earlier analysed by Dijkstra et al [8] and Carle et al [18] based on nuclear genes (18S rRNA, 28S rRNA) and mitochondrial genes (COI, 12S rRNA, 16S rRNA).

Conclusion

It has been concluded that mitochondrial COI gene is an effective tool for sorting out phylogenetic relationships among the closely related species. More species should be sequenced for better and reliable inferences to be drawn which can be used for classification of the family Platycnemididae. During the present study, COI gene sequences of 8 species (*Calicnemia eximia*, *Coeliccia chromothorax*, *Coeliccia fraseri*, *Coeliccia renifera*, *Disparoneura quadrimaculata*, *Esme cyaneovittata*, *Esme longistyla* and *Prodasineura verticalis*) have been submitted for the first time to NCBI, while sequences of 3 species (*Coeliccia didyma*, *Copera marginipes* and *Copera vittata*) have been submitted for the first time from India.

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