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## RESEARCH ARTICLE

# PHYLOGENETIC RELATIONSHIP OF SIX SPECIES OF PYRGOMORPHIDAE (ORTHOPTERA: CAELIFERA: ACRIDOMORPHA) AS REVEALED BY RAPD-PCR ANALYSIS

**[1,2]Jayashree, H. and [2,*]Channaveerappa, H.**

[1]Department of Biology, SDM College for Women, Mysuru-570004, India
[2]Research Group, Government Home Science College, Holenarasipura-573211, India

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**ABSTRACT**

Phylogenetic relationship of grasshoppers of family pyrgomorphidae is vague and less studied aspect, molecular analysis of phylogenetic relationships has not been done so far .In this study we have analysed phylogenetic relationships of six species of pyrgomorphidae using nuclear genome through RAPD-PCR.Ten primers used in this study have produced only polymorphic bands with varying numbers in between the species. The phylogenetic tree constructed based on similarity matrix depicts monophyletic origin of Pyrgomorphidae species and also predicts origin of congeneric species from different nodes.



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## INTRODUCTION

The family pyrgomorphidae includes grasshoppers with great morphological diversity between genus and species .Due to colorful markings on the body these grasshoppers are also known as gaudy grasshoppers. Over a long period of time substantial work has been done on the taxonomy of these grasshoppers and has resulted in different views in the placement of pyrgomorphidae among Acridomorpha. Species of pyrgomorphidae are distributed in Indopacific, Africa, Paleoarctic, Neotropic and Australian regions (Leavitt *et al*., 2013) consists of about 477 species in 149 genera. About 12 genera and 25 species of pyrgomorphidae have been analysed for taxonomic classification from Indian subcontinent on the basis of morphometric (Kirby, 1914) and genital structures (kumar *et al.,* 2013) and about 10 species of pyrgomorphids are posed for cytogenetic analysis (Rao1936,Channaverappa 1996) from this region but these studies have not revealed any species relationships with in pyrgomorphidae. Phylogenetic relationships of pyrogomorphidae is still a vague and less studied aspect. Phylogenetic relationships of pyrgomorphidae are analysed based on organization of male phallic structures but molecular phylogenetic relationships in between the members of pyrgomorphidae has not been done so far. In order to understand the relationship between the six species of Pyrgomorphidae, a molecular study based on RAPD-PCR analysis of nuclear genome has been carried out. Through this study phylogenetic relationships of the six species of this family is inferred.

****Corresponding author: Channaveerappa, H.***
*Research Group, Government Home Science College, Holenarsipura-573211, India.*

## MATERIALS AND METHODS

Six grasshopper species of pyrgomorphidae collected from their natural habitat were used for this study, these species include *Atractomorpha crenulata crenulata, Neorthacris acuticeps acuticeps, Crotogonusoxypterups, Chrotogonustrachypterus, Poecilocera picta, Pyrgomorphabispinosabispinosa.*

### DNA extraction

Total genomic DNA was extracted from all the 6 isolates using femur part of the leg. RAPD amplification (Wiliams *et al.,* 1990; Welsh and Mc clelland, 1990) was carried out using 10 random primers OPA-02, OPA-11,OPAA-09, OPAI-05,OPB-02, OPB-11,OPC-06, OPC-07, OPD-02, 970-11. According to

the protocol suggested by Sambrook *et al.* (1989) with slight modification using DNA kit (Aristogene, Bangaluru, India)

**RAPD-PCR**

The procedure described by Welsch and Mcclland (1990) and Willams *et al.* (1990) are adopted for analysis with slight modifications. RAPD-PCR was carried out in 40µl volume containing 17µl of D D $H_2O$,20µl of 2XPCR master mix, random primer1µl,template DNA2µl. Amplification was performed in Applied Biosystem PCR machine, gene amp 9600 of 0.2ml capacity. Programmed for an initial denaturation at $94^0$c for 5min then 40 cycles for 30sec at $94^0$c.1min annealing at $45^0$c of 40 cycles and 1.30min, extension at $72^0$c of 40 cycles, final extension of 7min at $72^0$c.Each PCR reaction was repeated twice in order to ensure that RAPD banding pattern were consistent and only stable products were scored. Amplified products were size fractioned using ladder marker (R-mid range ruler ranging from 0.1 to 3.5kbp) by electrophoresis in 2% agarose gels in 1XTAE buffer (Sambrook *et al.,* 1989) at 120v for 1hr. The bands were visualized by ethidium bromide under UV fluorescence and photographed. Ten random primers were used their DNA sequence are given in the list. The PCR protocol as adopted in the study resulted in reproducible pattern of amplicons using specific combinations of accession and primer only. The primer which displayed reproducible, scorable and clear band were considered for analysis The image profile of banding pattern were recorded. Gels were photographed by uvi tech USA. The gel profile were visually scored by assigning a number to each distinctive band.

**List of DNA primers**

| S. No | Primers | Sequence |
|---|---|---|
| 1 | 970-11 | GTAAGGCCG |
| 2 | Opa-o2 | TGCCGAGCTG |
| 3 | Opa-11 | CAATCGCCGT |
| 4 | Opaa-09 | AGATGGGCAG |
| 5 | Opai-o5 | GTCGTAGCGG |
| 6 | Opb-10 | CTGCTGGGAC |
| 7 | Opb-18 | CCACAGCAGT |
| 8 | Opc-06 | GAACGGACTC |
| 9 | Opc-07 | GTCCCGACGA |
| 10 | Opd-02 | GGACCCAACC |

The results were analysed based on the principle that a band is considered to be polymorphic if it is present in some individuals and absent in other and monomorphic if present in all the individuals or accessions. Total bands of polymorphism was evaluated,the number of bands generated per primer ranged from 0to 14.Distance and similarity matrix were calculated by Jaccoard's co-effient method to generate evolutionary tree by Neighbor joining method with Gelquest software version 1.0 to evaluate the evolutionary distance.

## RESULTS

RAPD-PCR reaction by 10 primers against 6 species of grasshoppers had generated 212 bands. All 212 bands were polymorphic, indicates large genetic variation in between these grasshoppers. The primer amplified DNA profiles is presented in Fig.1a. For all the primers used for 6 species of grasshoppers. All the 10 primers had generated bands with variance(Table1. a and b).

**Table 1a. RAPD-PCR bands polymorphism between six species of grasshoppers for 10 different primers**

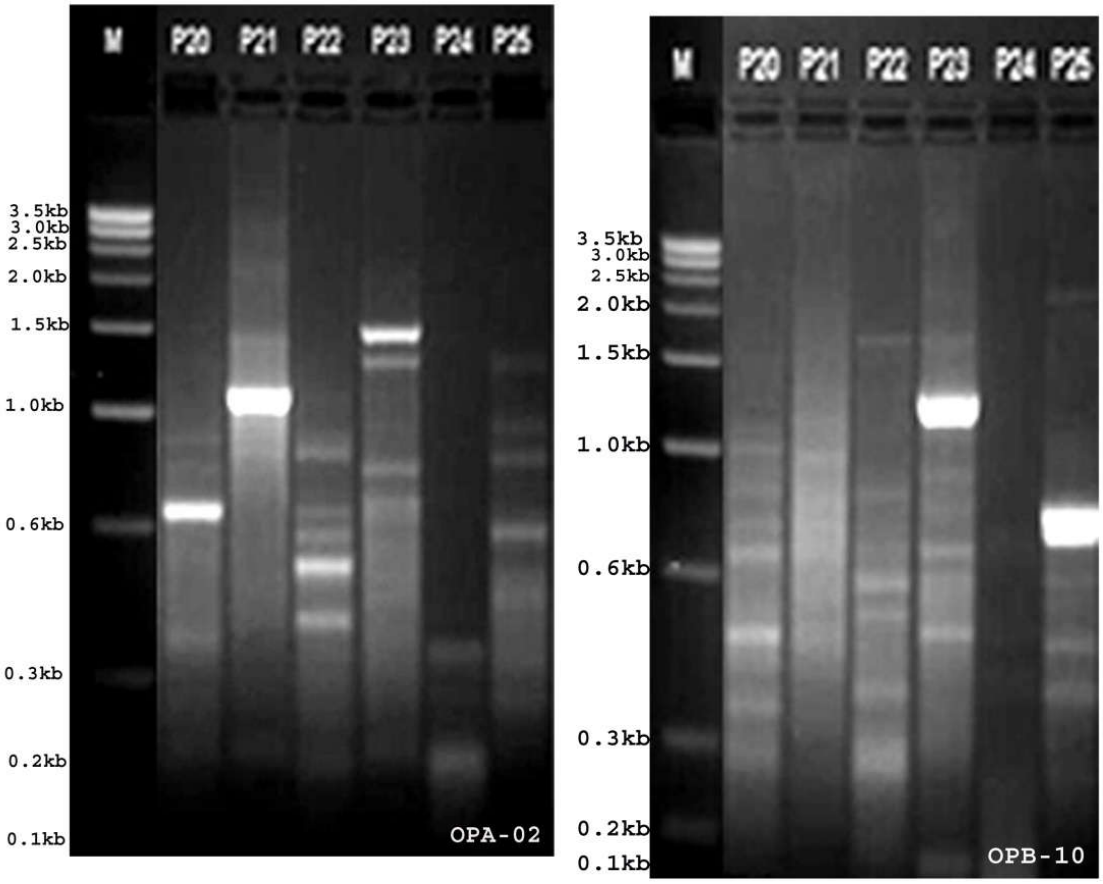
| Primers/species | P20 | P21 | P22 | P23 | P24 | P25 | TOTAL BANDS |
|---|---|---|---|---|---|---|---|
| OPA-02 | 4 | 2 | 4 | 4 | 2 | 3 | 19 |
| OPA-11 | 4 | 2 | 2 | 7 | 5 | 7 | 27 |
| OPAA-09 | 0 | 0 | 4 | 3 | 0 | 5 | 12 |
| OPAI-05 | 3 | 2 | 6 | 5 | 0 | 5 | 21 |
| OPB-10 | 7 | 2 | 4 | 4 | 0 | 4 | 21 |
| OPB-18 | 2 | 0 | 0 | 4 | 0 | 1 | 7 |
| OPC-06 | 11 | 10 | 6 | 7 | 1 | 6 | 41 |
| OPC-07 | 7 | 2 | 1 | 10 | 2 | 10 | 32 |
| OPD-02 | 5 | 4 | 6 | 8 | 1 | 1 | 25 |
| 970-11 | 1 | 2 | 1 | 3 | 0 | 0 | 7 |

Accession no:-
P20-*Atractomorpha crenulatacrenulata*. P21-*Chrotogonus oxypterus*
P22-*Chrotogonus trachypterus.* P23-*Neorthacris acuticepsacuticeps*.
P24-*Poecilocerapicta*P25-*Pyrgomorpha bispinosabispinosa*

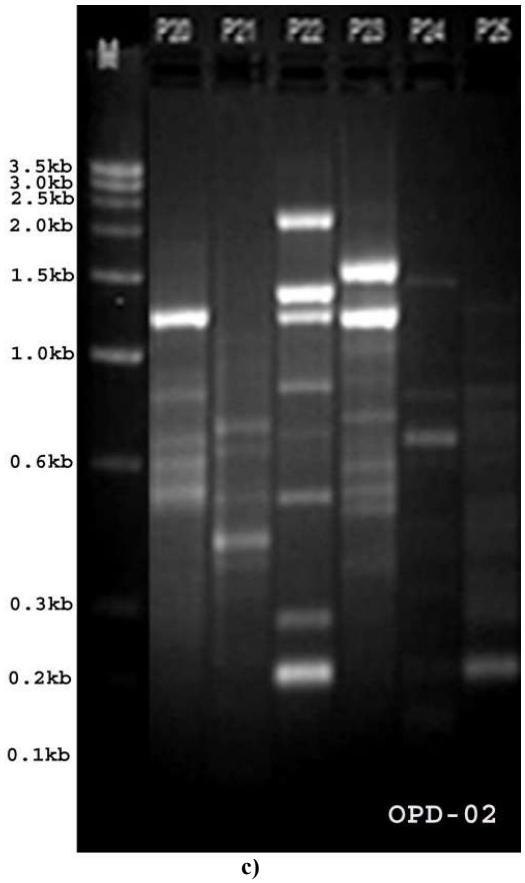
**Table 1b. Similarity & Distance Matrix for six species of grasshoppers**

| | P20 | P21 | P22 | P23 | P24 | P25 |
|---|---|---|---|---|---|---|
| P20' | 0 | 0.9181 | 0.92952 | 0.88837 | 0.88034 | 0.93162 |
| P21' | 0.9181 | 0 | 0.9148 | 0.92793 | 0.9125 | 0.90789 |
| P22' | 0.92952 | 0.9148 | 0 | 0.91509 | 0.8952 | 0.89954 |
| P23' | 0.88837 | 0.92793 | 0.91509 | 0 | 0.91739 | 0.91284 |
| P24' | 0.88034 | 0.9125 | 0.8952 | 0.91739 | 0 | 0.87446 |
| P25' | 0.93162 | 0.90789 | 0.89954 | 0.91284 | 0.87446 | 0 |

P20-*Atractomorpha crenulatacrenulata*.P21-*Chrotogonus oxypterus*
P22-*Chrotogonus trachypterus.* P23-*Neorthacris acuticepsacuticeps*.
P24-*Poecilocerapicta*P25-*Pyrgomorpha bispinosabispinosa.*




a)b)




c)

**Fig 1(a-c): RAPD Gel image profiles of the three primers**
**P20-*Atractomorpha crenulatacrenulata*. P21-*Chrotogonus oxypterus*, P22-*Chrotogonus trachypterus*. P23-*Neorthacris acuticepsacuticeps*, P24-*Poecilocerapicta*P25-*Pyrgomorpha bispinosabispinosa***

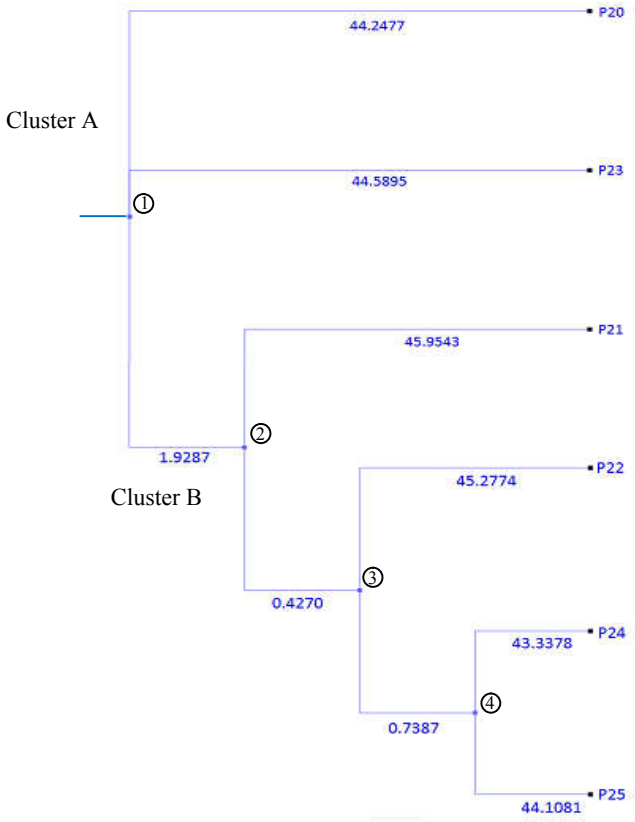



**Fig.2.a. Rooted phylogenetic Tree**

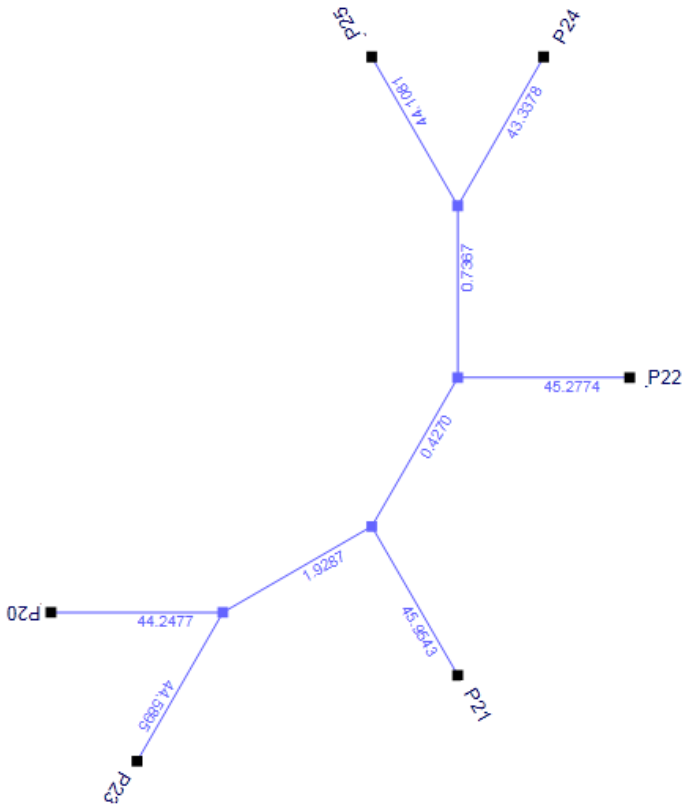



**Fig.2. b. Unrooted phylogenetic Tree**
**P20-*Atractomorpha crenulatacrenulata*.P21-*Chrotogonus oxypterus***
**P22-*Chrotogonus trachypterus.* P23-*Neorthacris acuticepsacuticeps*.**
**P24-*Poecilocerapicta*P25-*Pyrgomorpha bispinosabispinosa***

OPAA-09 did not generate bands in sample *A.c.crenulata, C.oxypterus, P.picta* OPAI-05&OPB-10 did not produce bands in sample P.pictus,OPB-18 did not produce bands in *P.picta, C.oxypterus* and *C.tracypterus* OPAI-05 andOPB-10 produced no bands for *P.picta*OPC-06 produced more number of bands with 41 of total bands, from all the 10 different primers. Similarly other primers generated bands ranging between 19-32.Polymorphism for bands profile is presented in Table-2. The result was subjected to cluster analysis which shows 2 main cluster A&B. Cluster A includes 2 species *Neorthacrisacuticepsacuticeps*&*Atractomorphacrenulatacrenulata*,with respect to distance values and cluster B has four species dividing further into 3 branches. Of these *Poecilocerapicta & Pyrgomorphabispinosabispinosa are closely related and Chrotogonusoxypterus & Chrotogonustrachypterus* are distantly related to each other. Even the unrooted tree profile shows *Poecilocerapicta & Pyrgomorphabispinosabispinosa* are closey related as well the other cluster includes two species, *Neorthacrisacuticepsacuticeps & Atractomorph acrenulata crenulata.*

## DISCUSSION

Orthopterans are one of the oldest group of extant insect lineages appeared in carboniferous era assume to have monophyletic origin (Song *et al*., 2015).This group includes caelifera, a sub order compounding Acridoidea and Phyrgomorphidae. There exists a lot of information regard to phylogeny of orthopterans in general but there is lack of molecular information to establish the relationships between the species and genera of Pyrgomorphidae. Attempts have been made to draw systematic or phylogenetic relationships between species of Pyrgomorphidae based on morphological characters like male genital structures (Dirsh 1956, Robert, 1941; Song, 2009 and 2010; Song and Bucheli 2010; Kumar *et al*., 2013) as well based on female genital structures (Usmani and Kumar, 2011). Phylogenetic relationships of Pyrgomorphidae with Acididae with in Acridomorphahas been analysed by several workers Roberts (1941) Flook and Rowel (1997), Eades (2000). Now it is placed as a single family Pyrgomorphidae under Pyrgomorphoidea (Kumar *et al*., 2013). Following inclusion of both Acrididae and Pyrgomorphidae as sister group that originate from a common ancestor Acridomorpha. All these relationships were drawn based on phallic structures as well molecular analysis (Leavitt *et al*., 2013), only in a few cases mt genome of 1-3 species of pyrgomorphidae were used with other species of acrididae to decide the phylogenetic position with other families of acridomorpha (Liu *et al*., 2008; Flook and Rowell 1997). Without contradicting the position of Pyrgomorphidae only the inter relationships of the six species of Pyrgomorphidae is examined in this study through RAPD-PCR analysis of nuclear genome. RAPD-PCR technique has been found to be more useful to draw phylogenetic or species relationships both in plants (Sairkar *et al*., 2010; Khan and Narayan 2007) and insects (Chapco *et al.,*1992) or genetic polymorphism in grasshoppers (JBT Dasilva *et al*., 2002) or analysis of paternity in grasshoppers (Kosal and Fever 1998). Use of RAPD technique for analysis of genetic diversity has been reviewed by several workers (Jain *et al*., 2010), (Basha sab *et al*., 2006), (Sentilkumarand G. Subramanya (2011), Bindroo and Moorthy 2014) and have recommended RAPD-PCR as one of the technique for molecular analysis for phylogenetic relationships. The application of this technique in this study has produced a good result to detect in between relationships of six species of Pyrgomorphidae. RAPD analysishas showed large genetic diversity with cognizable variations in morphology between six species of pyrgomorphids. The absence of monomorphic bands in this analysis from all the DNA primers indicategreater genetic diversity among these grasshoppers. The genetic distance found using RAPD analysis was between D=0.87446 to 0.93162among these species. The variations in terms of PCR product length >200 bp to 3500 bp and its frequency of insertion and deletion is sufficient for phylogenetic analysis. The phylogenetic tree for six species computed from similarity index values(Fig 2 a &b) has showed clear separation of the studied samples in two major clusters. RAPD analysis of phylogenetic relationships of six species of the family Pyrgomorphidae that are further categorised into five tribes diverge show monophyletic origin from a single root of the phylogenetic tree (Fig.2a and b). These six species diverge into two major clades A and B based on the similarity matrices. Clade 'A' includes *Atractomorpha crenulata crenulata*and *Neorthacris acuticeps acuticeps* that come under Atractomorphini and Orthocridini tribe respectively originating from a common node remaining four species *Chrotogonusoxypterus, Chrotogonus trachypterus (tribe: Chrotogonini). Poeceloceruspicta (tribe:*poiceloceni) and Pyrgomorphabispinosa (tribe:Pyrgomorphini) are closely related and monophyletic. Though *Chrotogonusoxypterus* and *Chrotogonus trachypterus* are monophyletic originate from two sub nodes.Infact species *A.c.crenulata* and *N.a.acuticeps* at the base of the phylogenetic treeindicate that these two species are relatively primitive within this group of six species of phyrgomorphidae. The species *C.trachypetrous* is basal to other remaining specieshad diverged later than *A.c.crenulata* and *N.a acutceps*. Two species *P.picta* and *P.bbispinosa* followed by *C oxypterous*, positioned at tip of the phylogenetic tree indicate their recent origin among the six species.

This study has revealed, the two major clusters diverged has shown relative relatedness among the clustered groups. Species in the cluster A have diverged early in the phylogeny are related to each other by 88.83%. the cluster B that includes four other species showed three ramifications at branch length 1.9287 giving rise to *Chrotogonusoxypterus*, followed by other two ramifications at branch length 0.4270 to *Chrotogonus trachypterus* and at length 0.7387 giving rise to *Poecilocerapicta* and *Pyrgomorpha bispinosa bispinosa* as the last ramification, indicating the recent origin. The species *Poecilocerapicta* and *Pyrgomorpha bispinosa bispinosa* are related to each other by 87.44% and these two with *Chrotogonus trachypterus* by 44.2%, likewise *Atractomorpha crenulata crenulata* and *Neorthacris acuticeps acuticeps* are related to *Chrotogonusoxypterus* by 43%, *Chrotogonusoxypterus* and *Chrotogonus trachypterus* are related to each other by about 43%, indicate greater diversity among these 6 species leading to phylogenetic ramification. In clade' B' inter species comparison of relationships between *Poecilocerapicta* and *Chrotogonusoxypterus* reflects as

polyphyetic groups and the tribe Chrotogonini is more closely related to the tribes Poicelocerini and pyrgomorphini based on the concept divergence from a node. In this study six species of grasshoppers show four divergence. A common divergence from the tree root node 1 and subsequent bifurcation into node 2 that generate node 3 includes the species of the genera Chrotogonus. Node 4 that originate from node 3 includes two morphological divergent species *Poeciloceraptera* and *Pyrgomorphabispinosabispinosa. T*hese two species have divergent food habit. *Poeciloceraptera* feed on highly toxic plant *calotropisgigantea* whereas *Pyrgomorpha bispinosa bispinosa* feeds on grass *Neorthacris acuticeps acuticeps* and *Atractomorphacrenuata* crenulata two have diverse morphology. Former is wingless feeds on mulberry, latter has wings feeds on grass, other two species *Chrotogonusoxypterus* and *Chrotogonus trachypterus* have similar body form with a few keycharacter differences but these two species originate from two different phylogenetic nodes as traced in this study . Such differences may be due to adaptive radiation driven by the evolution of key innovation .Key innovations were traits that provided a lineage access to a previously under exploited niche and associated resources (Funk and Nosil, 2007). This has to be examined further by comparative analytical approaches because speciation in animals is complex and heterogenous process. Such divergence in the morphology and food habit have no correlation with phylogenetic divergence of these species and may be due to later diversification.

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