

On the Occurrence of *Cheirotonus gestroi* (Scarabaeidae: Coleoptera) in Mizoram, Northeastern India: Molecular and Morphological Characterization

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ABSTRACT

Aim: This study aimed to validate the presence of *Cheirotonus gestroi* in Mizoram, North-eastern India, expanding its geographical distribution, and providing detailed morphological descriptions, particularly focusing on sexual dimorphism, for future identification. **Materials and Methods:** Specimens were collected from Murlen National Park (23.645°N 93.29222°E), Champhai district of Mizoram using light traps. After collection, the specimens were preserved in 70% alcohol for further analysis. The specimens were identified based on distinguishing morphological features and confirmed through molecular analysis by sequencing a partial segment of the mitochondrial 16S rRNA gene. **Results:** The results confirmed the presence of *Cheirotonus gestroi* in Mizoram, North-eastern India. This marks the first recorded instance of the species in Mizoram. Morphologically, *C. gestroi* was distinguished from other *Cheirotonus* species by having orange-spotted elytra, the male apical protibial spine longer than the median spine, and medially toothed, a feature that sets it apart from similar species in the region. Analysis of the 16S rRNA gene confirmed the identity of the species. **Conclusion:** This study confirms the presence of *Cheirotonus gestroi* in Mizoram, broadening its known distribution. The findings provide important morphological descriptions and sexual dimorphism in particular and further contribute to the understanding of the species' ecological significance. The molecular confirmation through 16S rRNA gene sequencing further enhances the accuracy of the species identification. The study also underscores the need for biodiversity surveys and highlights the importance of conservation efforts in protecting such species.

Keywords: Biodiversity hotspot, Genitalia, Occurrence, Sexual Dimorphism.

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INTRODUCTION

The Euchirinae, a subfamily of Scarabaeidae, usually comprise large-size beetles inhabiting mountain areas of Asia, the Middle East, and SE Europe.^[1,2] They are sometimes referred to as "long-armed scarabs" because of their exceptionally long forelegs.^[1] The subfamily Euchirinae is comprised of 16 species under three genera viz., *Propomacrus* Newman, 1837 (4 spp.), *Euchirus* Burmeister and Schaum, 1840 (2 spp.), and *Cheirotonus* Hope, 1840 (10 spp.).^[1,3-5] The montane highlands, which are heavily forested, are home to the Euchirinae because of the old-growth brood trees and plant exudates that immatures and adults feed on to survive.^[1]

The genus *Cheirotonus* can be distinguished from other Euchirinae species by its green background colour with shining pronotum, anterior edge of the prothorax protruding in the middle, and hairless anterior tibiae in males.^[1,6,7] Adults are huge with an intriguing metallic colour, enormous body size, and excessively lengthy arms (primarily males).^[8]

Species of *Cheirotonus* had been reported from the Khasi and Naga Hills, as well as the mountains of Manipur State, in Assam and along the Indian-Burmese border by Young^[1] and Ghosh *et al.*,^[9] but never from Mizoram. A recent Coleopteran survey conducted in Mizoram, North-east India resulted in the collection of a species belonging to the genus *Cheirotonus*. A detailed comparison of the species, using morphology and molecular approach, with other species revealed that it is *C. gestroi*, forming the first record from the region. The diagnosis and description provided here will help in the identification as well as revealing sexual dimorphism of the species, thus, helping in planning its conservation strategies.



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MATERIALS AND METHODS

Study site

Adult male and female *Cheirotonus gestroi* were collected from Murlen National Park (23.645°N 93.29222°E), Champhai district of Mizoram, India. Murlen National Park, situated on the border between Myanmar and India, spans an area of 200 square km. The Murlen sub-montane, semi-evergreen, and tropical forests, with its many cliffs, is well-known for being the habitat of numerous threatened bird species and other wild animals and plants.

Collection and preservation

A 160-watt mercury vapour lamp or bulb and a 20-Watt UV lamp (manufactured in Poland) were used as light sources to gather specimens at night using light traps in September 2023. The samples were kept in 70% ethanol till they were removed for morphological examination and brought to the laboratory at Pachhunga University College in Aizawl for further identification. Specimens' photography was taken against a white sheet of paper using a camera (Nikon D750). The abdomen was sliced open to remove the genitalia. Removed genitalia were washed and made pliable in a hot water basin. After that, it was washed in a 10% Potassium Hydroxide (KOH) hot water solution. Male genitalia were stored in a glass vial with 70% alcohol after being washed in 95% ethanol.^[10] Photographs of the genitalia were taken with a Leica S9i microscope.

Morphological identification

Morphological identification was based on available literature^[1,6] and the diagnostic characters include the length of the male median and apical protibial spines, the location of the terminal spines, pronotum punctures, and elytral spot patterns. All measurements, including the length of the male genitalia, were made point to point using a slide calliper to the nearest 0.1 mm.

Molecular identification

Phenol Chloroform Isoamyl Alcohol (PCI)^[11] was used to extract gDNA from soft tissue that had been preserved with ethanol. Extracted gDNA was used as a template to amplify the partial 16S rRNA mitochondrial gene.

The DNA amplification was done in a 25-μL reaction mix with Emerald Amp GT PCR Master Mix (Takara Bio, India) and 2 μL genomic DNA as a template. Partial 16s rRNA primers were used: forward (16SF13398-CGCCTGTTTATCAAAAACAT) and reverse (16SR12887-CTCCGGTTTGAAGTCAGATCA).^[12] The DNA amplification was performed using the ProFlex™ thermocycler (3x32-Well PCR System, Applied Biosystems) with the thermal regimes; 4 min at 95°C for initial denaturation, followed by 35 cycles of 50 sec at 94°C for second denaturation, 1 min for annealing at 54°C, 1 min of elongation at 72°C, and a final elongation for 7 min at 72°C. The PCR results were examined using 1.5% agarose gel electrophoresis.

The samples were then sequenced using Sanger's dideoxy technique on a sequencer (Eurofins Genomics India Pvt. Ltd., Bangalore, India.), with sequencing reactions performed solely in the forward direction. The generated sequences were uploaded to NCBI GenBank. The phylogenetic analysis utilised 23 nucleotide sequences from 22 Scarabaeidae taxa and one outgroup taxon, *Lucanus mazama* (KP250257). MEGA X was used for phylogenetic analysis.^[13] Clustal W was used to conduct the alignments with default parameter values, and pairwise distances were computed.^[14] The maximum likelihood tree was generated using HKY+G.

RESULTS

Systematic account

Family : Scarabaeidae Latreille, 1802,

Subfamily : Euchirinae Hope, 1840,

Tribe : Euchirini Hope, 1840,

Genus : *Cheirotonus* Hope, 1840,

Species : *Cheirotonus gestroi* Pouillaude, 1913,

Material examined: PUCZM/A/VIII/2301, 2 ex, 1 male and 1 female. India, Mizoram; Murlen National Park, 23.645°N 93.29222°E, 1,680m asl. The specimens were collected on July, 2023 by C. Zothansanga and T. Malsawmdawngzuali. The specimens are registered and housed at Zoological Museum, Department of Zoology, PUC.

Distribution: India: Manipur; Nagaland;^[1] Mizoram (New record). Elsewhere: Vietnam; Laos; Thailand; Myanmar;^[1] China.^[8]

Diagnosis

Cheirotonus gestroi is different from other continental Asian species by having orange-spotted elytra with the male protibial spine apically pointed, longer than the median spine and medially toothed. The species shared similar characters with *Cheirotonus battareli* Pouillade 1913, but markedly differs from it by having numerous large, deep, and dense punctures in the pronotum and having shining pronotal disc with numerous elytral spots which are often confluent.

Description

Male: Length 52.30 mm. Body large and heavy. Head and pronotum entirely brilliant shining green; elytra rarely unicolorous; marked with testaceous yellow, with varying patterns of orange spots across surface. Elytral orange spots numerous, often confluent, suggesting lines; Testaceous yellow spots on elytra abundant; clearly arranged in longitudinal series, sometimes confluent, increasing linear appearance; sutural edge underlined on each side of an interrupted testate line.

Head small, excavated anteriorly with a sigma-shaped elevation at middle of vertex, anterior margin entirely reflexed, rounded and with small denticles in dorsal aspect, broadly and rather arcuately produced downwards in frontal aspects; anterior margin of clypeus subtruncate with several small dentation on each side. Gula broad and arcuate. Surface strongly punctated with small erect hairs developing from the punctures. Punctures are rounded and irregular in size, not confluent with each other.

Transverse pronotum strongly narrowed posteriorly, 1.5 times (approx.) as broad as long, broadest immediately next to middle; sides obliquely expanded from posterior angles to broadest part, where they are broadly rounded and produced, and then strongly but somewhat arcuately attenuate to anterior angles, which are acute and sharply produced; anterior margin narrow, with a small obsolete but acute projection at the middle; posterior angles obtuse, produced; posterior margin bisinuate, with the median lobe broadly and accurately produced; lateral margin reflexed and irregularly denticulate, becomes obsolete and rather crenulate in the anterior half; disc broadly and strongly convex, with a broad, deep and oblique depression just before each posterior angle along posterior half of the margin, the convexity being bipartite by the median longitudinal groove, which is broader and deeper dorsally, shallower and obsolete anteriorly, closed posteriorly, and not reaching posterior margin; surface scattered with round punctures, but the punctuation becomes denser and somewhat confluent in the lateral depressions and the median groove, sparser and weaker on the convexities on each side of the median groove. Scutellum triangular, outer margin of the scutellum

dark green with inner region brilliant shining green and slightly rugose, anterior side punctured with few extending along the mid line.

Elytra 1.28 times (approx.) as long as wide, 2.4 times (approx.) as long as pronotum and broadest immediately next to middle; sides arcuately expanded at humeri, somewhat arcuately rounded from humeri to apices. Broad with the outer angles broadly and arcuately rounded and the sutural angles sub rectangular, very feebly produced; sutural margin broadly but obsolete elevated; lateral margins narrowly reflexed; disc convex, with basal depressions moderate, not deep and large, and with a faint trace of costa; surface glabrous, sparsely scattered with inconspicuous fine punctures and somewhat sparsely rugose on the lateral side.

Body beneath entirely clothed with suberect brownish-grey hairs with lesser and fine hairs in the middle of the last four sternites, except for those on the exterior side of prosternal episterna which are bright brownish, very long and dense, brush-like, and protrude from the sides of the pronotum and visible from above; pygidium broad, about twice as broad as long, invisible dorsally and clothed with semi recumbent hairs. Abdomen brilliant shining green with darker colour on the lateral side.

Anterior legs long, femora robust with the interior dentation large and the apical upper projection produced with a very extensive base, but abruptly detached towards the middle of its height at acute angle, fine denticulation present in the anterior half; tibiae long, slightly longer than tarsus and longer than femora, arcuate, with denticulation of the inner margin finer and irregular, outer edge of the tibia furnished with five sharp teeth, 4 tarsal claws

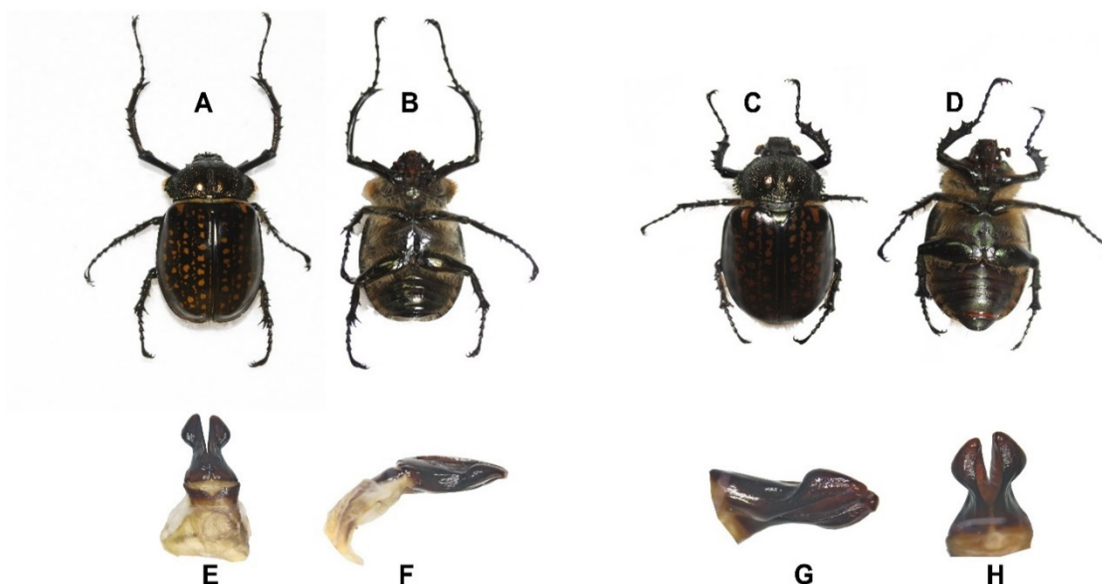


Figure 1: *Cheirotonous gestroi* showing A & B. Male (Dorsal and ventral view). C & D. Female (Dorsal & ventral view). E & F. Aedeagus (Dorsal and lateral view). G & H. Parameres (Ventro lateral view and ventral view).

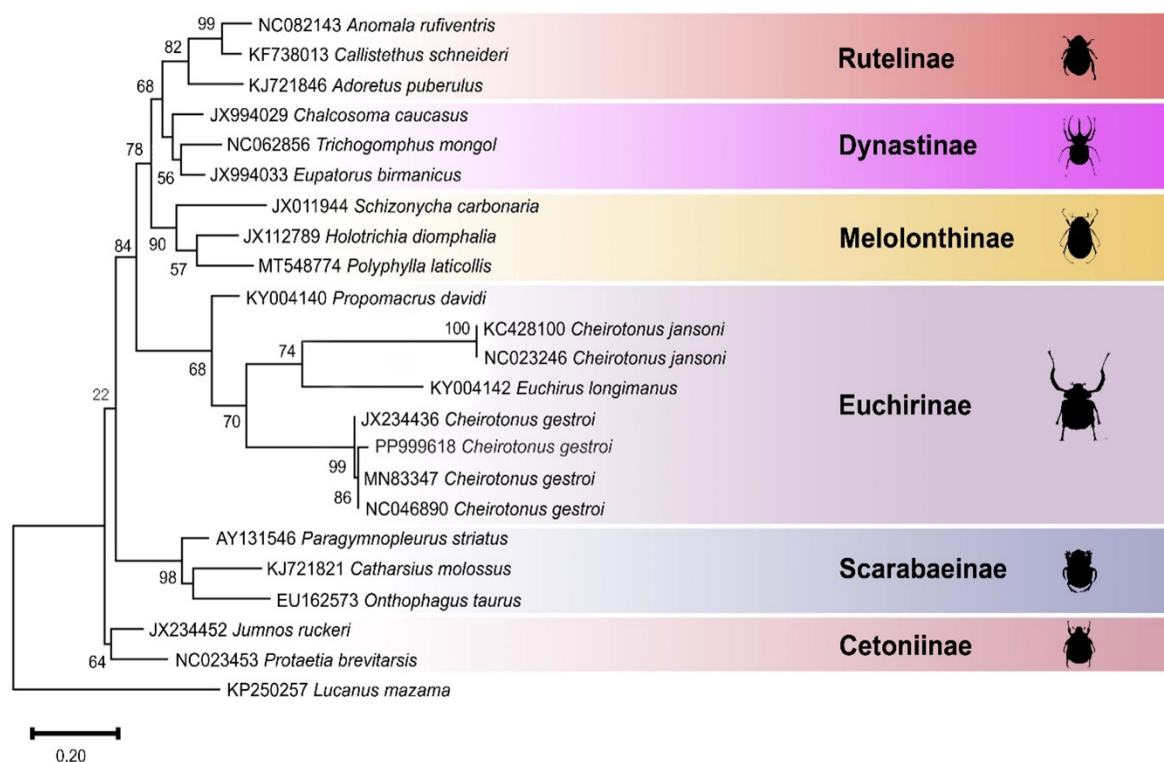


Figure 2: Maximum likelihood phylogenetic tree of *Cheirotonus gestroi* constructed based on the partial 16S rRNA gene and the comparison with its congeners retrieved from the NCBI database; number at nodes indicate bootstrap proportion support value (50% or more, 1000 replicates). Numbers before each species indicate the GenBank Accession number.

with two spurs projecting from it. The apical protibial spine longer than the median spine and medially toothed, not interiorly lined with dense hairs. Protarsus longer than both mesotarsus and metatarsus, mesotarsus longer than mesotibia and mesofemur and metatarsus longer than metatibia and metafemur. Mesotibia with 9 spines and coarsely punctured. Metafemur covered with hairs and denser in the anterior and posterior region when viewed dorsally, metatibia with 8 spines and finely punctured.

Female: Length 48.10 mm. Different from male in the following points:

Anterior pair of legs shorter and tibia slightly shorter than tarsus with no dentation on interior side. Protibial and median spine absent, with tibia more densely punctured in female. Apical upper projection absents in femur.

Clypeus truncated at anterior margin; broader than male.

Pronotum more rounded, 1.5 times (approx.) as broad as long, punctuation stronger, denser, and coarser.

Pubescence of body beneath weaker.

Pygidium exposed from elytra, 2 times (approx.) as wide as long, narrower with apex more sharply produced, hairs more brownish.

Male genitalia: Aedeagus length-7.68 mm. Large and robust, each paramere broad at base, depressed or sulcate, dilated laterally,

contracted and slightly curved inwards, pointed at apex (Figure 1).

Molecular characterization

Based on the analysis of mitochondrial 16S rRNA, the generated sequence of *C. gestroi* (NCBI Accession no: PP999618) exhibited 98.08% and 97.32% similarity (intra-species distance 1.9-2.6%) with the database sequence of *C. gestroi* from China (NCBI Accession no: MN893347 and NC046890) and *C. gestroi* from Laos (NCBI Accession no: JX234436). The only available sequence of other *Cheirotonus* species in the GenBank is *C. jansoni* (NCBI Accession no: NC023246 and KC428100) exhibiting 80.80% similarity with *C. gestroi*. Further, among the sub-family of Euchirinae, *Euchirus longimanus* (NCBI Accession no: KY004142) and *Propomacrus davidi* (NCBI Accession no: KY004140) are closer to *C. gestroi* (Figure 2).

Based on the maximum likelihood tree generated, each sub-family (Melolonthinae, Rutelinae, Cetoniinae, Scarabaeinae, Dynastinae and Euchirinae) formed a distinct clade through their representative genera obtained from the GenBank.

DISCUSSION

Most of the species in the genus *Cheirotonus* have been limited to highlands areas that experience a minimum of 1000 mm of annual rainfall because these regions, in general, provide

humus, mature broadleaf trees, and plant exudates which are essential for the sustenance of both larvae and adult insects.^[1] It is not surprising that *C. gestroi* occurred and was encountered in Mizoram, northeast India where the region is a subtropical moist broadleaf forest ecoregion. However, the few encountered rates of the species during the survey period may indicate the smaller population of the species.

Young^[1] and Šípek *et al.*,^[3] stated that eucharine species are often regarded as a bio-indicator of virgin, matured and established tropical forests. Furthermore, *Cheirotonus* sp. larvae are vital to the material cycle of forest ecosystems because they feed on the decaying wood residues of live trees.^[1,7] The life cycle of the species and its function in decomposition might be better understood with the help of future research on their feeding ecology, growth patterns, and developmental phases. Further research on *C. gestroi* adaptation to environmental changes (such as temperature, humidity, and habitat loss) may also be essential to forecasting the species' potential reactions to habitat modification and climate change. Destruction of habitat associated with anthropogenic activities such as plantation and the 'slash and burn' (jhum cultivation) method which is still very prevalent in these regions may lead to a drastic decline in the population of *C. gestroi*. Therefore, it is essential to implement scientific conservation and management approaches to guarantee the continuous existence of these species.

The morphological analysis in this study revealed that *Cheirotonus gestroi* is distinguished from its congeners in the male apical protibial spine, which is longer than the median spine and medially toothed, the shining pronotal disc with multiple large, deep punctures, and the orange areas on the elytra that frequently suggest lines. These diagnostic characters are in concordance with Young.^[1] It has been reported that the morphology of male genitalia is an important diagnostic character in identifying Coleopterans.^[1,15] The male genitalia, particularly the phallobase and the movable parameres, were examined and photographed to confirm the species' identity. The parameres of *C. gestroi* when viewed dorsally are broad at the base, dilated laterally and slightly curved inwards, pointed at the apex which differentiates it from other species.

In general, identifying morphologically similar species is always a challenge; the same also applies to many coleopteran species because of their morphological crypticity. Hence, many workers^[16-20] used and proved the reliability of 16S rRNA to delineate Coleopteran species. Although we hereby used a single marker (16S rRNA) for confirming *C. gestroi* identity, we opined that integrating data from multiple markers can give a detailed depiction of evolutionary relationships and increase accurate species identification.

The occurrence of *C. gestroi* in Mizoram, northeast India was partly anticipated given the species' prevalence in nearby states. This

documentation significantly broadens the species' distributional range. Subsequently, the provided detailed description including the sexual dimorphism among the species will help in further conservation of the species.

CONCLUSION

This finding significantly broadens the known distribution of the species, emphasizing the importance of current research and conservation initiatives. It also highlights the importance of integrating morphological and molecular methods of identification, particularly, in taxonomic studies to enhance the understanding of biodiversity. Moreover, the systematic study of *C. gestroi* within Mizoram, which is a portion of Indo-Burma biodiversity hotspot region, enhances the ecological understanding of its habitat and role within the ecosystem. The occurrence of this species emphasizes the need for ongoing biodiversity surveys to monitor and protect its habitat. Therefore, conservation efforts are essential to ensure the survival of species. Future studies on their effects on decomposition and forest health will help us better grasp their ecological relevance. Furthermore, a more comprehensive understanding of its behavior, genetics, and ecological role could not only contribute to the preservation of this remarkable species but also highlights the broader importance of conserving diverse ecosystems and the species they support.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

PUC: Pachhunga University College; **NCBI:** National Center for Biotechnology Information; **DNA:** Deoxyribonucleic Acid; **PCR:** Polymerase Chain Reaction; **PCI:** Phenol Chloroform Isoamyl Alcohol; **rRNA:** Ribosomal Ribonucleic Acid; **asl:** Above Sea Level; **KOH:** Potassium Hydroxide; **UV:** Ultraviolet; **IAEC:** Institutional Animal Ethics Committee; **DBT:** Department of Biotechnology; **NE:** Northeastern; **SE:** Southeastern; **ex:** Exemplars; **mm:** Millimeters; **MEGA:** Molecular Evolutionary Genetics Analysis; **HKY:** Hasegawa-Kishino-Yano (a molecular evolution model); **PUCZM:** Pachhunga University College Zoological Museum.

ETHICS APPROVAL

The work is approved (approval no. PUC-IAEC-2023-A14) by the Institutional Animal Ethics Committee (IAEC) of Pachhunga University College, Aizawl, Mizoram.

AUTHORS CONTRIBUTIONS

Chawngthu Zothansanga: Collect specimen, identified the specimen by molecular and morphological methods, conceived the idea, analyzed the data, and manuscript writing.

Tara Malsawmdawngzuali: Collect specimen, identified the specimen by molecular and morphological methods and analyzed the data.

Betsy Zodinpuui: Collect specimen, identified the specimen by molecular and morphological methods and analyzed the data.

Lalramliana: Conceived the idea, supervised the whole work and approved the final manuscript.

SUMMARY

The Euchirinae subfamily of Scarabaeidae comprises large beetles. These beetles are notable for their long forelegs and are found in tropical and subtropical regions. The occurrence of *C. gestroi* in Mizoram, India, is reported in this paper. Male genitalia were characterised and detailed morphological descriptions were given. To validate the identity of *C. gestroi*, molecular identification was performed using a single marker (16S rRNA). However, we believe that combining data from numerous markers can lead to a more thorough knowledge of evolutionary connections and increase the accuracy of species identification. This study offers Mizoram's first documentation of *C. gestroi*. The comprehensive characterisation of *C. gestroi* and its traits will facilitate the identification of sexual dimorphism. Additionally, this paper elucidates the significance of biodiversity conservation and the need for scientific conservation and management techniques to ensure the long-term survival of these species.

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