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Research Article

Morphological description and molecular DNA barcoding of a new record of earthworm *Travoscolides chengannures* from Odisha, India

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ABSTRACT

Biodiversity finds importance in ecosystem functions and soil biota finds particular significance in nutrient and organic matter recycling. Earthworms are known to play a major role in contributing to soil fertility and participate in diverse ecosystem functions and services. Thus study of their biodiversity and conservation finds importance. In the present study, earthworm specimens of *Travoscolides chengannures* (*T. chengannures*) were collected from Bhubaneswar, Odisha, India for morphological species detection and further molecular confirmation to resolve complex from closely related species using cytochrome oxidase gene (COI gene) as fewer gene sequences available for this species. This species from the State of Odisha, India, is the new record. For the amplification of COI gene, universal primers LCO1490 and HCO2198 were used. Our morphological study revealed new records of *T. chengannures* and COI sequences (accession numbers: OM536159, OM536160 and OM536161) showed close affinity among each other, but formed a separate cluster with other strains of this species. This confirms the prevalence of species complex in this species which needs more efforts to resolve it.

Key words: *Travoscolides chengannures,* cytochrome oxidase gene (COI gene), Odisha

INTRODUCTION

Charles Darwin, in 1881, the great naturalist, observed and highlighted the importance of earthworms in increasing soil fertility. They are known to thrive in soil even in harsh conditions in the soil being exposed to external environmental stress factors like pollutants, radiations and pathogens due to their robust immune system (Ghosh, 2018, 2020 a, b, c, 2021). Earthworms are known as the friends of the farmers due to their activity in the soil, and recently a lot of research is being conducted on the earthworm gut microbial community (Szmigiel *et al.,* 2021). This bacterial community is known to trigger earthworm immunity (Dvořák *et al.,* 2015).

With 3700 reported species of earthworms globally, more than 400 species under 70 genera have been reported from India; mostly characterized based on of morphological taxonomic features (Giller *et al.,* 1997, Julka and Paliwal 2005, Julka *et al.,* 2009) indicates the biodiversity of earthworms. Recently in 2022, Ghosh *et al.,* published an extensive checklist on Indian earthworms (Ghosh *et al.,* 2022a) revealing the pan India biodiversity of earthworm.

Earthworms are largely studied by their anatomical and morphological features along with the dissection of the anterior end of adult worms. However, earthworm taxonomy suffers from various limitations *i.e.* overlapping features within taxa, unidentifiable life stages of other than that of adult forms, variable anatomical and morphological features of similar taxa leading to ambiguity in identification of a species. The application of molecular markers of DNA barcoding finds importance in solving such ambiguity and enable proper identification of species (Folmer *et al.,* 1994, Pop *et al.,* 2003, Chen *et al.,* 2011).

Travoscolides chengannures is an important species of earthworm for many soil functions, however, studied not well at genetic level and fewer mitochondrial cytochrome oxidase I (mt-COI) sequences available in genbank also (https://www.ncbi.nlm.nih.gov/nuccore). The earlier sequences showed significant variations among them. We collected this species from Odisha, India for the first time. Therefore, we have proposed DNA barcoding with mt COI for identification and resolve species complex of *T. chengannures* along with morphologic features.

Since there are natural forces like climate change, pathogen, radiation exposure, man made threats like pollution that threaten the biodiversity, life, biology, reproduction, of earthworms, it is important to catalogue and study earthworm distribution time to time across different places of a country(Ghosh 2018, 2021, 2022b).

Studies, based to detect the conservation status of earthworm are usually neglected (Marchán *et al.,* 2022) across the globe and many species are on the verge of extinction. There are not many reports from the country. Therefore this study of new records of *Travoscolides chengannures* from the state of Orissa, with insights into the genetic diversity finds importance in highlighting the importance of conservation of earthworms in India.

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MATERIALS AND METHODS

Earthworm sampling

Earthworm specimens were collected in 2021 from different areas of Bhubaneswar, 20.2961° N, 85.8245° E, Odisha, India (Fig 1) during the monsoon months of June-July. Soil pits (15 × 15 × 15 cm) dug with a spade was used to collect the earthworms by hand-sorting and collected specimens were preserved in 70% ethanol and brought back to the laboratory for morphological identification (Kushwaha *et al.,* 2016, Gates, 1940) and for molecular species identification. A total of 3 specimens were used in the study.

DNA extraction, Polymerase Chain Reaction (PCR), and sequencing

Molecular studies comprising of extraction of DNA and their amplification for COI gene was carried out as in (Folmer *et al.,* 1994). Specimens were washed in distilled water. Then tissue samples comprising of muscular body wall were taken from clitellum encompassing the tail tip and was preserved in 100% alcohol. Genomic DNA was isolated using MP biomedical kit using standard procedure. Isolated DNA was amplified with LCO & HCO universal forward and reverse primers (from IDT,USA) generating aDNAfragment of approximately 650 bp, respectively. PCR was performed in a reaction volume of 25 µL containing 1X Taq polymerase buffer with1.5mmol·L –1 of $MgCl_2$ (Genei, India), 100 µL·mol·L –1 of each dNTP (Thermo), 0.5 pmol of forwardandreverse primers, 50-100 ng of genomic DNA and 1.0 U of Taq DNA polymerase (Genei, India). PCRamplifications were performed in a thermal cycler (Biorad, USA). The thermal conditions for LCO & HCO primer pair were 95˚C for pre-denaturation, 3min (onecycle); 95˚C for denaturation, 45 sec, annealing 58˚C, 45 Sec, extension 72˚C, 1 min (30cycles); final extension 72˚C, 10 min (one cycle). The amplified products were resolved in 1.5%agarosegel, stained with ethidium bromide (EtBr, 10 mg/ml) and gel was visualized by gel documentation system. DNA was eluted in 200 µl preheated elution buffer and stored at -20ºC until further analysis. The purified amplicons were sequenced by Sanger's method of sequencing. The sequencing results were assembled and compared with NCBI databases.

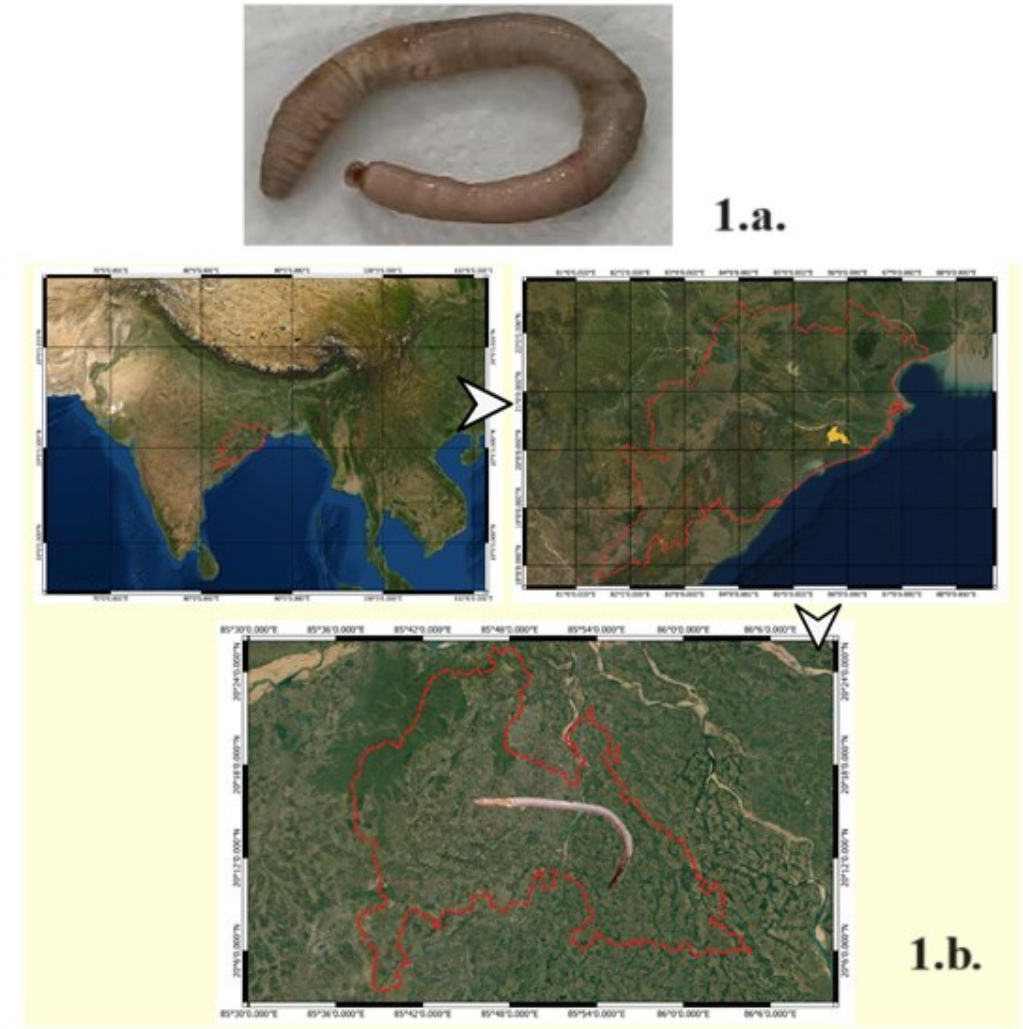

Figure 1. a. *Travoscolides chengannures* (external form); b. Map of sample collecting site of Odisha, Bhubaneswar (20° 56' 27.3120" N, 84° 48' 12.4812" E)

Phylogenetic relationship using Mt-COI gene sequence

The good quality sequencing reads were aligned and manually edited using MEGA-X software and compared to existing sequences in the Genbank database (NCBI). The sequences generated here and their best match in the existing databases were exported into MEGA-X for alignment. All COI sequences generated were submitted to the GenBank database (accession numbers: OM536159, OM536160 and OM536161).

Twelve (12) partial genome sequences of earthworm were downloaded from GenBank (https://www.ncbi.nlm.nih.gov/GenBank) using the blastn search tool along with the three (3) sequenced earthworm samples. *Bacillus subtilis* strain sequence used as an outer group comparison. The sequences were aligned alongside the sequence data generated from this study using MAFFT v. 7 and BioEdit software. From ZSI Kolkata, phyletic affiliations for COI sequences were constructed using maximum likelihood statistical method and the pairwise genetic distance was estimated based on the Kimura-2-parameter substitution model (Kimura 1980) and Nearest-Neighbor-interchange heuristic method taking bootstrapped 1000 (Suman *et al*. 2021, 2022).

RESULTS AND DISCUSSION

Taxonomic identification

In a recent checklist of study of earthworms on a pan India distribution, we reported 32 valid species from the state of Odisha, India (Ghosh *et al.,* 2022). From this study we report 33 valid species reported now from the state of Odisha, India. There is a possibility that the soil pH and the components of the soil are indicative about the limited geographic dispersal of the identified species as they were never recorded from the state of Odisha, India (Singh *et al.,* 2020).

Our morphological study revealed new records of *Travoscolides chengannures* (*T chengannures*) for Odisha state. COI sequences (accession numbers: OM536159, OM536160 and OM536161) showed close affinity with other strains of this species confirming the species. *Travoscolides*. Gen. Nov.was originally identified from Travancore and Cochin (1949–1956), which later in 1956 were included under Madras state of India and are distinguished from other Indian species. Diagnosis includes male pores with combined apertures of prostatic ducts and penisetal follicles on xviii segment. Female pores paired on xiv segment. Quadrithecal,

Tables 1. Details of mitochondrial cytochrome c oxidase subunit I (COX1) gene sequences of *Travoscolides chengannures* used in this study.

Species	GenBank Accession number	Location	GPS Location
Travoscolides chengannures	OM536159.1	Odisha, India	
Travoscolides chengannures	OM536160.1	Odisha, India	20° 56' 27.3120" N 84° 48' 12.4812" E
Travoscolides chengannures	OM536161.1	Odisha, India	
Octochaetona beatrix	MN175259.1	North East India	25.5736° N, 93.2473° E
Travoscolides chengannures	MT410728.1	North East India	
Travoscolides chengannures	KX832079.1	Madhya Pradesh, India	23° 28' 23.9664" N and 77° 56' 52.7928" E
Travoscolides chengannures	MT396228.1	North East India	25.5736° N, 93.2473° E
Travoscolides chengannures	JN185606.1	Trivandrum, Kerala	8° 31' 26.9004" N 76° 56' 11.8968" E
Travoscolides chengannures	MT410730.1	North East India	
Travoscolides chengannures	MT410729. 1	North East India	
Travoscolides chengannures	MT410728.1	North East, India	25.5736° N, 93.2473° E
Travoscolides chengannures	MT396229.1	North East, India	
Travoscolides chengannures	MT396228.1	North East, India	
Travoscolides chengannures	MT380474.1	North East, India	
Bacillus subtilis strain B3 polyketide synthase (pks) gene, partial cds	MW027660.1	Kerala, India	10° 51' 1.8576" N 76° 16' 15.8880" E

spermathecal pores on or near 7/8-8/9. All reproductive apertures minute and superficial. Clitellum annular reveal inginter segmental furrows, dorsal pores and setae. First dorsal pore is observed on 11/12 segment. Setae lumbricine with ventral setae of xviii segment, penial and in closely paired follicles. Prostomium observed to be prolobous. Pigmentation septa present from 5/6 segment. Gizzard in v segment. Calciferous glands four pairs in x-xiii segments, which appearreniform, concave side medially, reaching below level of ventral face of gut and medially, opening by short and slender stalks from ventral ends into gut slightly lateral to mid-ventralline. Intestine begins in xv segment. Typhlosole simple, lamelliform located from xxiv-xxv to lxivcl segment. Hearts latero-oesophageal in x-xiii segment. Supra-, extra-oesophageal and lateroparietal trunks observed. Excretory organs appear closed, revealing enteronephric or exonephric form. Micro nephridia observed in vertically placed clusters on anterior faces of 5/6-13/14 segments, from xv posteriorly closed, (enteronephric or exonephric) micro nephridia on posterior faces of the septa. Holandric, seminal vesicles observed in xi-xii segments. Prostates observed to be tubular. Male deferent ducts opening into ental ends of prostatic ducts.Spermathecae with elongated sausage-shaped ampullae and a short ducts, diverticulum disc-shaped, shortly stalked and with arborescent lumen.

Mt-COI gene sequence and phylectic tree analysis

GenBank accession OM536159, OM536160 and OM536161 (https://www.ncbi.nlm.nih.gov/nuccore/?term=OM536159:OM536161(accn)) with descriptions of the sequences from earlier studies are shown in the phylogenetic tree (Fig 2). The maximum likelihood tree inferred from *COI* gene DNA sequences revealed the phylogenetic position of the specimen.

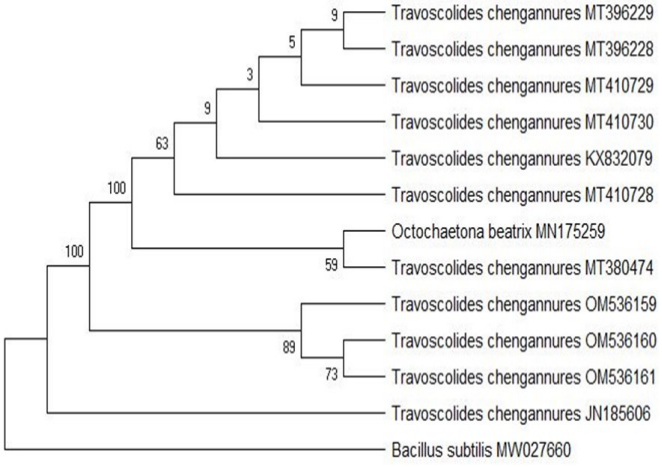

Figure 2. Phylogenetic tree of three earthworm samples of *Travoscolideschengannures*. Phylogenetic analysis of *Travoscolides chengannures* with GenBank accession OM536159 (A), OM536160 (B) and (C) OM536161are shown in the phylogenetic tree. (D) Phylogenetic tree through Bootstrap method of the three earthworm samples. Out group used here is *Bacillus subtilis*. .(E) Phylogenetic tree through Bootstrap method of the three earthworm samples, calculated in a order of 500 cycles from the root.

Despite some previous studies on the taxonomy of earthworms have been done globally, imperative knowledge gaps regarding the ecological status and molecular phylogenetic analysis of several earthworm species requires in-depth investigation. Due to difficulties regarding proper identification of these ecologically important group, comparison of diverse species based on the essential ecosystem services provided by them is tedious and therefore needs proper species identification and inventorization.

Our present study highlights the existence of species of earthworm *T. chengannures* studied both morphologically and with the assistance of DNA barcoding using COI gene, being reported for the first time from the state of Odisha in India. During the recent years, the emergence of molecular taxonomy based on DNA barcoding approach has efficaciously assisted comparison and biodiversity assessment of diverse species of invertebrates including earthworms, identification of new as well as old taxa based on reference materials available online in several databases like NCBI GenBank, phylogenetic reconstruction (King *et al.,* 2008; Chang & James 2011; Decaëns *et al.,* 2013). In this study, the phylogenetic tree showed multiple clusters of *T. chengannures.* The north eastern Indian and Kerala state populations of this species showing difference in COI gene sequences from this present strain from Odisha. The phylectic analysis also suggests that there are possibilities of prevalence of species complex in this species which is hard to detect morphologically. Hence, we suggests further studies for better resolution of complex present in *T. chengannures.*

CONCLUSION

The earthworm *T. chengannures* collected from Bhubaneswar, Odisha, India is the new record for this state. Although in 2022, our group published an extensive pan India work highlighting the biodiversity of Indian earthworm (Ghosh et al., 2022a), this study further confirms the biodiversity of earthworms in India adding this new record from this state of India, hereto unreported. The COI gene revealed close affinity among all the three individuals of this species, but they deviate significantly from other locations specimens collected from north eastern states of India, Kerala and Madhya Pradesh suggesting prevalence of species complex.

Earthworms by their capacity to decompose organic matter by microbial communities inhabiting their digestive track, contributing to the drilosphere has been reported to affect soil processes, plant growth and play role in nutrient cycling including N_2 recycling by modifications of the microbial community. (Butt & Lowe 2011) and therefore conservation measures for this vital organism gains profound importance.

Although studies at the modern times elucidate, the importance of earthworms in contributing to soil fertility, very little has been studied regarding the ecology of earthworm, relations and interaction with soil condition and their microbial communities from India.

Despite some previous studies on the taxonomy of earthworms have been done globally, imperative knowledge gaps regarding the ecological status and molecular phylogenetic analysis of several earthworm species and their conservation measures requires in-depth investigation. Due to difficulties regarding proper identification of these ecologically important group, comparison of diverse species based on the essential ecosystem services provided by them is tedious and therefore needs proper species identification and inventorization.

As compared to other common earthworm species, *Travoscolides chengannures* has been reported and identified with concrete morpho-taxonomic and molecular taxonomic data for the first time from the state of Odisha, India. Thus, this study would facilitate earthworm biologists to focus further on evaluating its relationship with other native species to generate a baseline data and to compare species diversity and dispersal.

There is a possibility that the soil pH and the components of the soil are indicative about the limited geographic dispersal of the identified species as they were never recorded from the state of Odisha, India.

Studies of distribution and biology of *Travoscolides chengannures* are extremely few and their relation and interaction with soil remains largely an unexplored domain. Besides more studies are underway to understand the complexity of species complex and their role and interaction with soil and improving soil fertility which remains our future scope of work.

Although a global data on earthworm diversity has been reported recently, (Phillips *et al.,* 2021) and they are known to play vital role in ecosystem functions and services including adding of soil fertility, decomposition, nutrient cycling, climate regulation, bioindicators of soil biodiversity and health, studies, on their conservation status are globally neglected (Marchán *et al.,* 2022) with many species are on the verge of extinction across the globe. There are also not many reports from the country.

Therefore, the current study of *Travoscolides chengannures* from across India, and the new records from the state of Orissa, with insights into the genetic diversity finds importance in highlighting the importance of conservation of earthworms in India.

DECLARATIONS

Ethics Approval: No vertebrate or human tissues were used. Earthworms are invertebrates, collected from local soil and recorded under Zoological Survey of India, Kolkata following approved protocols under ZSI India, Kolkata were studied.

Consent to Participate: NA

Consent to Publish: All authors consent to publish

Authors Contributions: SG conceived the study, SG, SD, NH, CM did the experiments, analyzed the data, wrote the paper. SG, RB, SD and DB revised the manuscript.

Completing interest: None declared

Availability of data and materials : All data are available

Funding: No funding was received for the study

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