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Identification of selected earthworm species of Northeast India using DNA barcoding

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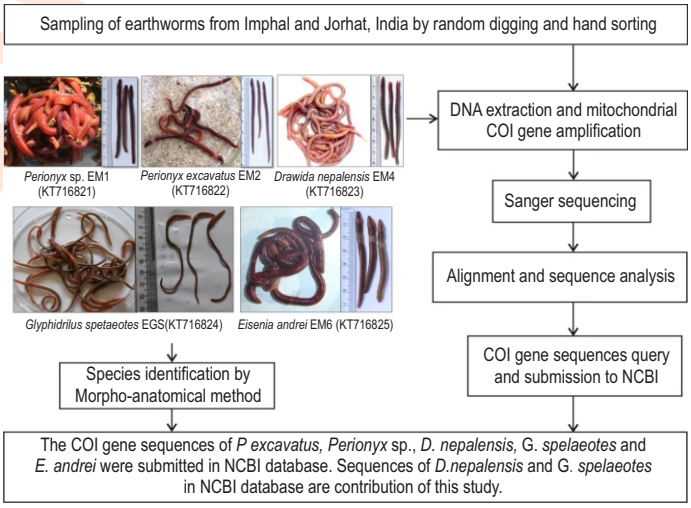
Abstract

Aim: This study aimed at the species identification of selected indigenous earthworms of Manipur and Assam, Northeast India along with an exotic species using morpho-anatomical study and DNA barcoding.

Methodology: Indigenous species of earthworms were collected from Imphal and Jorhat, North-eastern part of India. The exotic species of earthworm were collected from Indian Council of Agricultural Research Complex, Manipur. The samples were collected by digging and hand sorting method. Identification of samples was done by both conventional and molecular methods. Molecular characterization was accomplished through PCR amplification of the mitochondrial cytochrome oxidase I (COI) genes. Automatic sequencing reactions were performed for the amplified PCR products on ABI3100 Genetic Analyser (Applied Biosystems).



Results: Out of five specimens (EM1, EM2, EM4, EG5 and EM6) examined through morpho-anatomical studies, three were identified to species level while the other two were identified to their genus level only. Out of EM1 and EM2 specimens in the genus *Perionyx* as per the morpho-anatomical studies, DNA barcoding could deduce the EM2 specimen up to the species level as *P. excavatus*. The exotic EM6 specimen morphologically identified as *Eisenia fetida* showed 99% COI gene sequence similarity with both *E. fetida* and *E. andrei* but its sequence divergence with *E. andrei* was less than 1%, so, it belonged to *E. andrei*.

Interpretation: This study shows the reliability of clubbing DNA barcoding experiments with classical taxonomy in supplementing and strengthening the traditional taxonomy for accurate identification of earthworms.

Key words: Earthworms, Mitochondrial cytochrome oxidase I gene, Morpho-anatomical studies, Species identification

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Introduction

The diversity of soil animal communities encompasses a large part of terrestrial ecosystems but yet it is poorly understood since there is a strong taxonomic impediment suffered by the most groups of animals in soil biota (Rougerie *et al.,* 2009). Earthworm is the major component of soil invertebrates and the key group of detritivores in the soil, often referred as ecosystem engineers (Cameron *et al.,* 2016). They are used as important model organisms for testing toxicity of chemicals in the soil ecosystems (Rombke *et al.,* 2016). Understanding the role of earthworms in ecosystem functions and their effective use in ecotoxicological testing requires precise identification of the species so that experimental data are interpreted accurately. However, limited information is available on variation in global diversity of earthworms. Several classical taxonomic studies on earthworm species have been carried out in different parts of India. Earthworm diversity has been reported by several researchers from different parts of North Eastern Region (NER) of India like Meghalaya (Lone *et al*., 2020), Manipur (Thounaojam and Singh 2020), Mizoram (Lalthanzara *et al.,* 2020), Assam (Rajkhowa *et al*., 2014) and Tripura (Chaudhuri and Bhattacharjee, 2005). However, little is known about the earthworm diversity, distribution pattern and community structure of earthworms across NER of India which lies in the Indo-Burma mega biodiversity hotspot. Exhaustive explorations are required in order to understand the diversity and distribution of earthworms in the NER of India.

Identification of earthworm species based on classical method suffers many drawbacks since this method is time consuming, labour intensive and require a high level of taxonomic expertise for the non-specialist to use the taxonomic keys for the correct species identification. Moreover, it is also not possible to identify juvenile earthworms by using conventional method. These discrepancies can be overcomed by generating genetic data using molecular approach of DNA barcoding (Rougerie *et al.,* 2009). The DNA barcode is a powerful and reliable tool for discrimination of species in earthworms and provides a useful complement to morphology-based classical taxonomy (Voua Otomo *et al.,* 2009). Several research groups from various parts of the world initiated focusing on the use of molecular techniques for species identification but the development of earthworm sequence database is especially confined in Europe and the United States. There has been strong interest in data poor developing countries including India for contribution to the global database like NCBI on earthworm sequences. Recently, very few systematic studies on earthworm have been carried out pertaining to DNA barcoding in India.

Various molecular markers such as Restriction Fragment Length Polymorphism (RFLP), Random Amplified Polymorphic DNA (RAPD) and Simple Sequence Repeats (SSR) are used in assessing genetic diversity but the most highly sequenced marker within earthworms is the mitochondrial cytochrome oxidase I (COI) gene. It has proved to be effective for species identification and revealing cryptic speciation. DNA barcoding works related to earthworms of India is at its initial stage. To assess the genetic diversity of Indian earthworms, Meenatchi *et al.* (2009) and Giraddi *et al.* (2009) used RAPD-PCR method for the first time. Mathur *et al.* (2010), Sharma *et al.* (2011) and Biruntha *et al.* (2013) did preliminary molecular works on earthworms of India. Rao *et al.* (2014) studied phylogenetic studies of *Eudrilus eugeniae* by sequencing the 18S rRNA gene. Kumar *et al.* (2013) reported earthworm species identification through sequencing of the 16S rRNA gene from Tamil Nadu, South India, while Jaya *et al.* (2015) and Kushwaha *et al.* (2015) evaluated the COI gene sequences for earthworms of Kerala, India. Recently, Lone *et al.* (2020), Samrendra *et al.* (2020) and Lalthanzara *et al.* (2020) adopted integrative approaches of morpho-anatomical taxonomy and COI gene sequences using DNA barcoding technique to identify earthworm taxa from Meghalaya and Arunachal Pradesh, India.

The diversity of earthworm species found in the Imphal district of Manipur and subtropical climatic condition of Manipur have been evaluated by Haokip and Singh (2012, 2017) and Thounaojam and Singh (2020). The earthworm species from Assam, India was reported by Rajkhowa *et al.* (2014). However, they did not employ molecular approaches and consequently, no DNA barcodes for these reported species have been generated. New COI gene sequences were expected to emerge from unexplored areas of NER of India. It has been suggested that DNA barcodes should be used with the integration of morpho-anatomical study. Consequently, the present study aimed to identify four native earthworms (three species from Imphal, Manipur and one from Jorhat, Assam) of NER of India along with an exotic species by integrating conventional method with COI gene sequencing.

Materials and Methods

Collection of earthworms: Three native mature species of earthworms were collected from soil, cowdung heap and banana pseudostem in Imphal whereas one aquatic species was collected from submerged rice field in Jorhat. The exotic earthworm was collected from ICAR-NEH Manipur Centre. Earthworm species were labelled EM1, EM2, EM4, EG5 and EM6.

Morphological identification of earthworms: The collected earthworms were washed several times with tap water and sterile water. For each earthworm species, three clitellate individuals were used for identification. The specimens were anesthetized with 30% ethanol solution. For each individual, a small sample of tissue (approximately 300 mg) was taken from the posterior region and stored in 95% ethanol at ambient temperature until DNA extraction (Huang *et al.,* 2007). The remains of each specimen were fixed in 10% formaldehyde and were sent to Julka cottage, Himachal Pradesh and the Zoological Survey of India, Kolkata for their taxonomic identification. The voucher specimens were maintained in IBSD, Imphal West, Manipur.

DNA extraction, PCR and sequencing: In order to validate the results obtained from the morpho-anatomical studies on identification of five earthworms, the molecular technique of PCR amplification and the COI gene sequencing were employed. Genomic DNA was extracted from three individuals per species following a standard phenol/chloroform extraction method (Huang *et al.,* 2007). Prior to PCR, DNA was quantified spectrophotometrically to dilute the genomic DNA to 50 ng μl^{-1}. DNA samples were stored at -20°C until further processing. The COI gene was amplified using the flanking primers LCO1490 (5'-GGTCAACAAATCATAAAGATATTGG-3'-) and HCO2198 (5'-TAAACTTCAGGGTGACCAAAAAATCA-3') (Folmer *et al.,* 1994). PCR cocktails (50 µl) contained 5 µl of PCR buffer (10x) with 15 mM $MgCl_2$ (Genei, KT03), 1.0 µl of 10 mM dNTP (Promega, U151A), 1.0 µl of each primer (0.01mM), 0.50 µl of Taq DNA polymerase (5 $U\mu l^{-1}$) (Genei, 232071IA), 1.0 µl of 25 mM $MgCl_2$ (Genei, 105881), 1.0 µl of 20 ng template DNA and 39.5 µl of sterile deionised water. Negative control without any tissue DNA was included in each PCR assay.

Amplification was performed in the thermal cycler (1000R Bio-Rad, USA) according to the thermocycling conditions given by Huang *et al.* (2007). The amplified PCR products were quantified in 1% agarose gel stained with Ethidium Bromide using 0.5xTBE buffer for 90 min at 70V. The 100bp DNA ladder (GeNei, RMBD135) was included for estimating the size of DNA fragments. The gel was observed using the ChemiDoc MP Gel documentation system (Bio-Rad, Hercules, USA). Automatic sequencing reactions were performed for the amplified PCR products on ABI3100 Genetic Analyser (Applied Biosystems) using the same primers used for amplification. The alignment of the nucleotide sequences was done using ClustalW implemented in BioEdit 7.0.5.3 using the default settings and were then edited manually. All sequence alignments were pruned to 587 bp. The sequences obtained were deposited in the Genbank database of NCBI. Neighbor-Joining (NJ) analysis was performed using MEGA6 with Kimura-2-Parameter (K2P) model (Kimura, 1980). Bootstrapping was performed with 1000 pseudo-replicates. Genetic distances were calculated using MEGA6 based on K2P model.

Results and Discussion

Out of five specimens examined through morpho-anatomical studies, three were identified to species level while the other two were identified to their genus level only. EM1 and EM2 showed characteristics of *Perionyx* genus but had apparent differences in their morphological features and yet their species status could not be assigned based on morphological study. The exotic species (EM6) obtained from ICAR-NEH Manipur Centre, the aquatic species (EG5) and the soil inhabiting earthworm (EM4) were identified as *Eisenia fetida* (Savigny, 1826), *Glyphidrilus spelaeotes* (Stephenson, 1924) and *Drawida nepalensis* (Michaelsen,1907), respectively through morpho-anatomical studies. The occurrence of *G. spelaeotes* and *D. nepalensis* was reported in another North-eastern state (Chaudhuri and Bhattacharjee, 2005). However, COI gene sequences of none of these earthworm species samples from North-east India have been determined and submitted in any database. The Genbank accession numbers of the COI gene sequences of all the five species were assigned from KT716821-25 and MT472583-92. Three (EM1, EM2 and EM 6) out of five specimens showed great similarity with the sequences of BOLDSYSTEMS and NCBI databases. On the other hand, the query COI gene sequences of two specimens, EM4 and EG5, which were identified as *D. nepalensis* and *G. spelaeotes,* respectively by morphol-anatomical study did not match with any sequences available in BOLDSYSTEMS and NCBI database. The COI gene sequences of several species under *Drawida* genus are available in the database (Blakemore *et al.,* 2014) and non-match of COI gene sequences suggests that *Drawida* species of this study is genetically different from others. A recent study reported that the molecular data were more useful for comparison of two species, *D. ghilarovi* and *D. ganini* within *Drawida* genus (Zhang *et al.,* 2021). Thus, the COI gene sequences of *D. nepalensis* and *G. spelaeotes* were made available in NCBI database for the first time from this study and this would facilitate comparison of sequences of similar species likely to be isolated from unexplored habitats in the future.

Out of EM1 and EM2 specimens in the genus *Perionyx*, EM2 showed 99% similarity with *P. excavatus* (EF494507, JX908692, EWSJA657-08), while EM1 showed 99% similarity with *Perionyx* sp. (HM219172, EWSJA831-08). The COI gene sequence of the specimen (EM6) collected from ICAR-NEH Manipur Centre which was taxonomically identified as *E. fetida* showed 99% similarity with both *E. fetida* and *E. andrei*. The mean genetic distances of this specimen with *E. fetida* 1 (AY874513, EF077596) and *E. fetida* 2 (AY874515, JX531571) were found to be 16.8% and 16.6%, respectively, while with that of *E. andrei* (AY874512, JX912899, LC278598) was found to be 0.58% only. *E. fetida* 1 and *E. fetida* 2 represented one single haplotype (Rombke *et al.,* 2016). The neighbour joining tree showing relationship between five different earthworm species and reference sequences is represented in Fig. 1. The phylogenetic analysis showed that the sequence of the exotic earthworm (EM6) maintained in IBSD was identified as *E. fetida* by the conventional method grouped with sequences of *E. andrei*. The newly submitted sequences of *D. nepalensis* and *G. spelaeotes* grouped with other species of *Drawida* (*D. gracilis* and *D. hattamimizu*) and *Glyphidrilus* (*G. peninsularis, G.singaporensis* and *G. kotatinggi*) genus, respectively. The sequences of two earthworms of the *Perionyx genus*, EM1 and Em2 were grouped with *Perionyx* sp. and *P. excavatus*, respectively.

In recent times, molecular methods have emerged and been useful in valid identification and in strengthening the result of classical taxonomy of earthworms. In this study, confirmation and accurate identification of the earthworms was achieved by employing a combination of classical taxonomy and DNA barcoding. Although DNA barcodes cannot completely replace classical taxonomy, it has been used as a powerful tool in

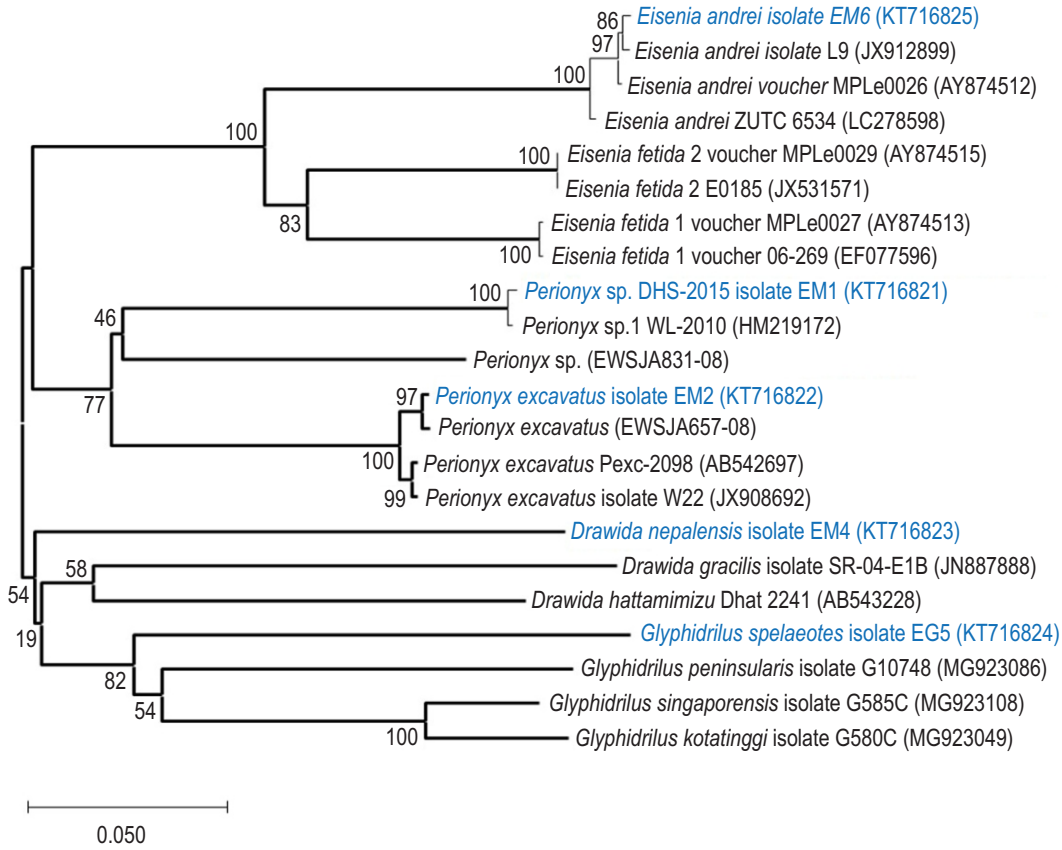

Fig 1: Neighbour-joining tree based upon COI gene sequences of five earthworm species showing relationship with reference sequences obtained from NCBI databases. GenBank accession numbers are shown in parenthesis after the species name. Numbers at nodes indicate bootstrap values from 1000 replicates.

resolving earthworm morphotypes and taxonomic dilemma of interspecific, intraspecific and cryptic species. Its use in creating database for the rich diversity of earthworms from the different regions of India has been limited. In NCBI database, COI gene sequences are mostly from America and Europe (Cameron *et al.,* 2016). This is the first report of COI gene sequences of four native earthworm species from Imphal, Manipur and Jorhat, Assam, India. Out of the four native earthworm specimens, the COI gene sequences of two earthworms EM4 and EG5, identified through classical taxonomy as *D. nepalensis* and *G. spelaeotes*, respectively did not show the exact or nearest matches with any species data available in the BOLDSYSTEMS and NCBI database. Therefore, the COI gene sequences of these two species were submitted for the first time in the NCBI database and are currently available as reference sequences in future.

The occurrence of *D. nepalensis* in the Koirengei Forest of Manipur has already been reported by Haokip and Singh (2017) and that of *G. spelaeotes* in Assam by Chanabun *et al.* (2013) and Dey *et al.* (2018). *G. spelaeotes* was also reported from Meghalaya and Tripura (Tiwari *et al.,* 2020). The two earthworm specimens EM1 and EM2 could not be identified to species level by classical taxonomy. However, DNA barcoding could deduce the morphological diagnosis of the EM2 specimen up to the species level as *P. excavatus* since similar COI gene sequences were already available in the NCBI and BOLDSYSTEMS database.

The identification of EM6 specimen based on classical taxonomy results of this study and comparison with COI gene sequence data of NCBI and BOLDSYSTEMS has raised controversy. It was further analyzed through comparison of their genetic distances with the already published reference sequences of NCBI database. Several researchers (Huang *et al.,* 2007, Voua Otomo *et al.,* 2009 and Pérez-Losada *et al.,* 2005) have reported the sequence divergence between species greater than 15% in most cases. The EM6 specimen taxonomically identified as *E. fetida* showed 99% similarity with both *E. fetida* and *E. andrei*. But the mean genetic distance of this species with *E. andrei* (AY874512, JX912899, LC278598) was found to be 0.58% and it could be accepted as *E. andrei*. *E. fetida* and *E. andrei* are considered to be cryptic species (Voua Otomo *et al.,* 2009). *E. fetida* is characterized by the presence of pale or yellow transverse segmental stripes, while *E. andrei* corresponds to the

uniformly reddish morphs. Except for the differences in pigmentation, these two species were similar in their morphology (Reinecke and Viljoen, 1991). After fixing the specimen in 10% formalin, the characteristic color of the segments disappeared. Sims (1983) and Bundy *et al.* (2002) reported that since the characteristic yellow transverse segmental stripes of *E. fetida* disappeared after fixation, the only difference in the morphological appearance was often rendered insufficient to identify these worms correctly. Rombke *et al.* (2016) reported that *E. andrei* was never erroneously identified as *E. fetida*, while *E. fetida* was often misidentified as *E. andrei*. Dhakane and Shinde (2020) also reported that the identification and differentiation success of *E. fetida* was 96.42%, whereas, it was 100% for *E. andrei*. Since the specimen EM6 used in this study did not show striped or banded morph, it belonged to *E. andrei*.

This was further supported by the NJ tree (Fig. 1) which depicted that this specimen formed a common clade with reference sequences of *E. andrei* while the *E. fetida* 1 and *E. fetida* 2 sequences formed two distinct clades. A similar result of the formation of three clusters had been reported with respect to *E. fetida* / *E. andrei* complex (Voua Otomo *et al.,* 2009; Rombke *et al.,* 2016). Thus, based on our data, the exotic earthworm (EM6) stock obtained from ICAR-NEH Manipur Centre, Imphal and maintained in IBSD was identified correctly in this study. This will facilitate correct interpretation and comparison of data on different characteristics of this earthworm species which is of high practical significance. Fiftyone species of exotic earthworms were recorded from India. *E. fetida* and *E. veneta* are in the list, however, *E. andrei* is not enlisted (Julka 2014). *E. fetida* was in the checklist of earthworm species of the NER of India (Assam, Sikkim and West Bengal) as reported by Tiwari *et al.* (2020).

So far 14 species (*D. nepalensis*, *Pontoscolex corethrurus, Dichogaster bolaui*, *Eutyphoeus gammiei*, *E. manipurensis*, *Amynthas cortices*, *A. gracilis*, *A. morrisi*, *Kanchuria sumerianus, Metaphire anomala, M. houlleti, M. birmanica, P. excavatus, P. shillongenisis*) have been reported from the Manipur state (Stephenson 1921; Haokip and Singh, 2012, 2017; Thounaojam and Singh, 2020). However, all these works were purely based on conventional method of identification. Genetic data were not generated in these studies. In the present study, the COI gene sequences of two earthworms (*D. nepalensis* and *G. spelaeotes*) out of four native earthworm species were submitted to NCBI database for the first time. This study shows the reliability of clubbing DNA barcoding experiments with morpho-anatomical study in supplementing and strengthening the classical taxonomy for accurate identification of earthworms.

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Add-on Information

Authors' contribution: S.D. Hijam: Field work, experiment execution, data analysis and manuscript preparation; **R.S. Thounaojam:** Field work and manuscript preparation; **M.C. Kalita:** Assisted in editing manuscript; **N.C. Talukdar:** Conceptualization of experiment and Mentor of the project.

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