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DNA barcoding of earthworms from lateritic semi evergreen forest of *Kolli* hill, a part of Eastern Ghats, Tamil Nadu, India

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Abstract

Approximately 8,300 species of earthworms have been described in Oligochaetes with 38 families and 811 genera in the world. Earthworms are considered as friends of farmers and hold them in high esteem as nature's ploughmen. The species identification is essential to know their ecology and life cycle. However, the species identification of adult earthworms is possible by dissection of the male genitalia but this method is labor intensive, time consuming and very difficult for non-specialists. The Cytochrome C Oxidase I (COI) gene is present in all animals. Thus, the COI gene defined as the DNA barcode has been used to identify species of earthworms. The collected earthworms form lateritic semi evergreen forests of Kolli hill, a part of Eastern Ghats, Tamil Nadu, India and were subjected to sequence analysis of COI gene and the same was deposited in GenBank. The data was further analysed.

The results and conclusion of this research declared that the evolutionary divergence of the D. gracilis, D. bullata, H. stuarti , M. cochinensis and P. corethrurus showed variation; the inter species variation was also observed in some species that may due to the environmental factor of the study area. It is obvious that the COI sequence of the above cited earthworm species may be used for the identification of this species reported from any part of the world through BLAST analysis if the identical sequences are submitted to GenBank in future.

Keywords: Earthworms, Kolli Hill, COI, DNA Barcoding.

Introduction

Earthworms, important ecosystem engineers[8,34], constitute up to 90% of the soil invertebrate biomass. Approximately 8,300 species of earthworms have been described (of which more than 5,700 are valid species) in Oligochaetes with 38 families and 811 genera in the world.[23] Conventionally, the earthworms are identified up to species level by using a combination of both morphological and anatomical traits described by taxonomists. Species identification of adult earthworms is possible by dissection of the male genitalia[28,35]; however, this method is labor intensive, time consuming and very difficult for non-specialists, particularly when dealing with field collections consisting of several different earthworm species.

Furthermore, identification is limited to adult worms, as most life stages are unidentifiable and many morphological and anatomical characteristics of earthworms have high degree of variability and overlapping features between taxa.[22] Hence, numerical taxonomic, the only possible criteria would vary between researchers to identify the earthworm. For example, the number and location of male pores are very important in earthworm taxonomy because these characteristics are related to copulation and reproduction. Male pores in section XVIII were once used to define families of earthworms[3]; however, this was discontinued in subsequent studies because pore location was found to vary among families and even within a single family.[18,31]

Due to the advancement in the molecular taxonomy which could over vent the prevailing problems in the numerical taxonomy, the species identification becomes more authentic. Huang et al[16] were the pioneers to envision the broader application of DNA barcoding for species identification and discrimination. Anticipate that DNA barcoding techniques will be increasingly used by ecologists in the near future. They will be able not only to identify a single species from a specimen or an organism's remains but also determine the species composition of environmental samples. Mitochondrial DNA (*mt*DNA) has been widely used in phylogenetic studies of animals because it evolves much more rapidly than nuclear DNA, thereby resulting in the accumulation of differences between closely related species.[5,19,20]

A 658 bp fragment of the mitochondrial gene cytochrome c oxidase I (COI) has been proposed as a standard barcode for animal species.[12] Sequence divergence is much higher among different species than within species, and *mt*DNA genealogies generally capture the biological discontinuities recognized by taxonomists as species. The COI gene is present in all animals, and sequence comparisons are straightforward because insertions and deletions are rare.

Thus, the COI gene has been defined as the DNA barcode for animals by Hebert et al[12] and is used to identify species of birds[14], springtails[15], spiders[2], tropical Lepidoptera[11] and insect pests[1] including invasive leaf miners.[26] Although this

method (DNA barcode) is not without controversy[17], the taxonomic problems with earthworms are serious enough that it is critical to seek a novel solution. Critics of DNA barcoding object to abandoning the use of traditional morphological characteristics and argue that relying on a single mitochondrial gene region for identification can be misleading, particularly because of widespread mitochondrial polyphyly/paraphyly.[30,36]

However, DNA barcoding method could be performed at any life stage of an animal in general and earthworms in particular; indeed, reliable identifications of juvenile or even partial specimens are possible[21,32], when identification of the same is not possible through observations on the morphology. A DNA barcode system will be helpful for immediate applications. Furthermore, the development of a universal DNA-based identification system could provide a globally important tool for the identification earthworm species. In earthworms, a very close match was found between traditional taxonomic identifications of closely related species and barcode clusters for Taiwanese species, suggesting that these genetic data would reliably identify species and point out cases of cryptic diversity.[6]

The aim of the present study is to determine the comparison between the molecular sequencing between the earthworms with reference to the morphological characters by using Cytochrome c oxidase I (COI) collected from lateritic semi evergreen forest of Kolli Hill.

Material and Methods

The sampling area from lateritic semi evergreen forest is located in Kolli hill, a part of Eastern Ghats, Tamil Nadu, India. The entire Kolli hill occupies 503 ha and of them the present study area of Lateritic semi evergreen forest spreads on 2058 ha which contributes about 8% of the total forest area of Kolli hills which geographically lies between 11^0 10'00'' to 11^0 30'00''N and 78^0 15'00'to 78^0 30'00'E. Maximum elevation of Lateritic semi ever green forest is 1200 m and the minimum elevation is of 800 m. After locating twenty sampling sites randomly spread across Lateritic semi evergreen forest, they were visited once in every month for a two year period to study and find out the earthworm diversity.

Earthworms were collected by digging and hand sorting method from twenty quadrats each with the dimension of 25cm x 25 cm x 40 cm depths as suggested by Senapathi and Sahu.[27] For each species, 5 to 8 adult earthworms were preserved in 99% ethanol at ambient temperature for later DNA extraction. Samples were taken from caudal tissue to prevent contamination by gut contents. The anterior part of each earthworm was kept in 100% ethanol at Centre for Eco-friendly Agro-Technologies, Research Department of Zoology, Nehru Memorial College (Autonomous), Puthanampatti, Tamil Nadu, India for identification of the same animals up to species level by conventional methods. Genomic DNA was isolated from 10-30 mg of the tissues using DNeasy® Blood and Tissue Kit (Qiagen) by following manufacturer's instructions. The quality of the isolated DNA was checked using agarose gel electrophoresis.

PCR amplification reactions were carried out in a 20 µl reaction volume which contained 1X PCR buffer (100mM Tris HCl, pH-8.3; 500mM KCl; and 0.01% gelatin), 0.2mM each dNTPs (dATP, dGTP, dCTP and dTTP), 2.5mM $MgCl_2$, 20ng DNA, 1 unit of AmpliTaq Gold DNA polymerase enzyme, 0.1 mg/ml BSA and 4% DMSO, 5pM of forward primer LCO – GGTCAACAAAT CATAAAGATATTGG and reverse primer HCO-TAAACTTCAGGGTGACCAAAAAATCA.[10] The PCR amplification was carried out in a PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems). PCR amplification profile for COX1 95ºC -5.00 min, 95ºC-0.30 min, 45ºC-0.30 min and 72ºC-0.30 min for10 cycles, 95ºC-0.30 min, 51ºC-0.30 min and 72ºC-0.30 min for 30 cycles,72ºC- 7.00 min was carried at 4ºC.

The PCR products were checked in 1.2% agarose gels prepared in 0.5X TBE buffer containing 0.5 µg/ml ethidium bromide. Specially formulated buffer for the removal of unwanted primers and dNTPs from a PCR product mixture with no interference in downstream applications was incubated at 37ºC for 15 min followed by enzyme inactivation at 80ºC for 15 min.

Sequencing was done in a PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems) using the BigDye Terminator v3.1 Cycle sequencing Kit (Applied Biosystems, USA) following manufacturer's protocol. The sequence quality was checked using Sequence Scanner Software v1 (Applied Biosystems).

All sequences were submitted to Genbank (Accession Numbers: JN036370, JN793516, JN793517, JN793518, JN793519, JN793527, JN793528, JN887887, JN887888, JN887889, JN887890, JN887891, JN887892, JN887893, JN887894, JN887895, JN887896, JN887897 and JN887898). Most similar sequences were retrieved from GenBank (Accession Numbers: HQ529284, GU013837, AB542550, AB542549, AB546862, AB542475, AB542527 and AB542528) with the submitted sequence through BLAST search. All sequences were aligned using Clustal W and UPGMA analysis, neighbor-joining tree[25] and maximum parsimony tree analysis.[9] Estimates of evolutionary divergence between sequences were performed with Mega 5.[33]

Results

There were five different earthworm species belonging to four families collected from Lateritic Semi Evergreen Forests of Kolli hills namely *Drawida gracilis, Drawida bullata* (Moniligastridae), *Hoplochaetella stuarti* (Octochaetidae), *Megascolex cochinensis* (Megascolecidae) and *Pontoscolex corethrurus* (Glossoscolecidae) and we generated sequences from 19 adult earthworms from these

species. Three to Four barcode sequences were obtained for all species. Each of 11 species belongs to six families included in our Neighbor-Joining profile tree possessing distinct COI sequences (Fig. 1-3) and none were shared between species. For clarity, each species was listed once and the number of individuals is in brackets (Fig. 1-3).

The results show that COI sequences of the species are most similar or identical to other sequences of the same species in all individuals. The sequences which we retrieved from Gen-Bank as allied species clustered taxonomically with their similar sequenced species. The three trees had similar topology; that is to say, DNA barcoding can be applied in identifying earthworms up to species level.

The estimated value of the shape parameter for the discrete Gamma Distribution is 0.2805. Substitution pattern and rates were estimated under the General Time Reversible model (+G). A discrete gamma distribution was used to model evolutionary rate differences among sites (5 categories, [+*G*]). Mean evolutionary rates in these categories were 0.00, 0.04, 0.23, 0.87 and 3.86 substitutions per site. The nucleotide frequencies are A = 27.64 %, T/U = 30.05 %, C = 23.36 %, and G = 18.95 %. For estimating maximum likelihood values, a user-specified topology was used.

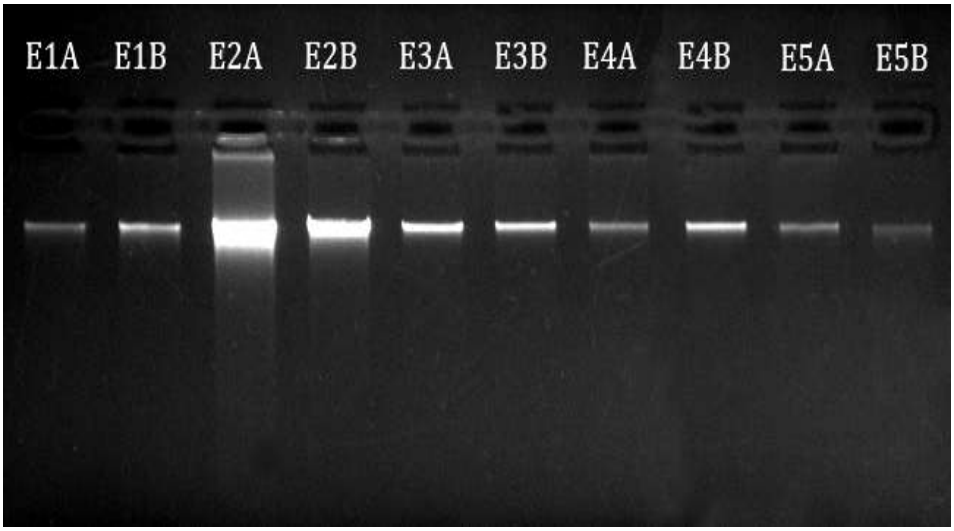

Fig. 1: Results of DNA extraction from collected earthworm species

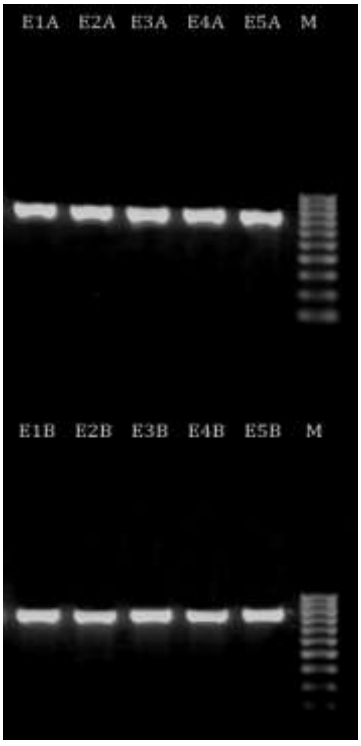

Fig. 2: Results of PCR products of earthworm species (COX1) M: 100 bp ladder (E1A, E1B- *D. gracili*, E2A, E2B - *H. stuarti*, E3A, E3B - *M. cochinensis*, E4A, E4B - *D. bullata* and E5A, E5B - *P. corethrurus*)

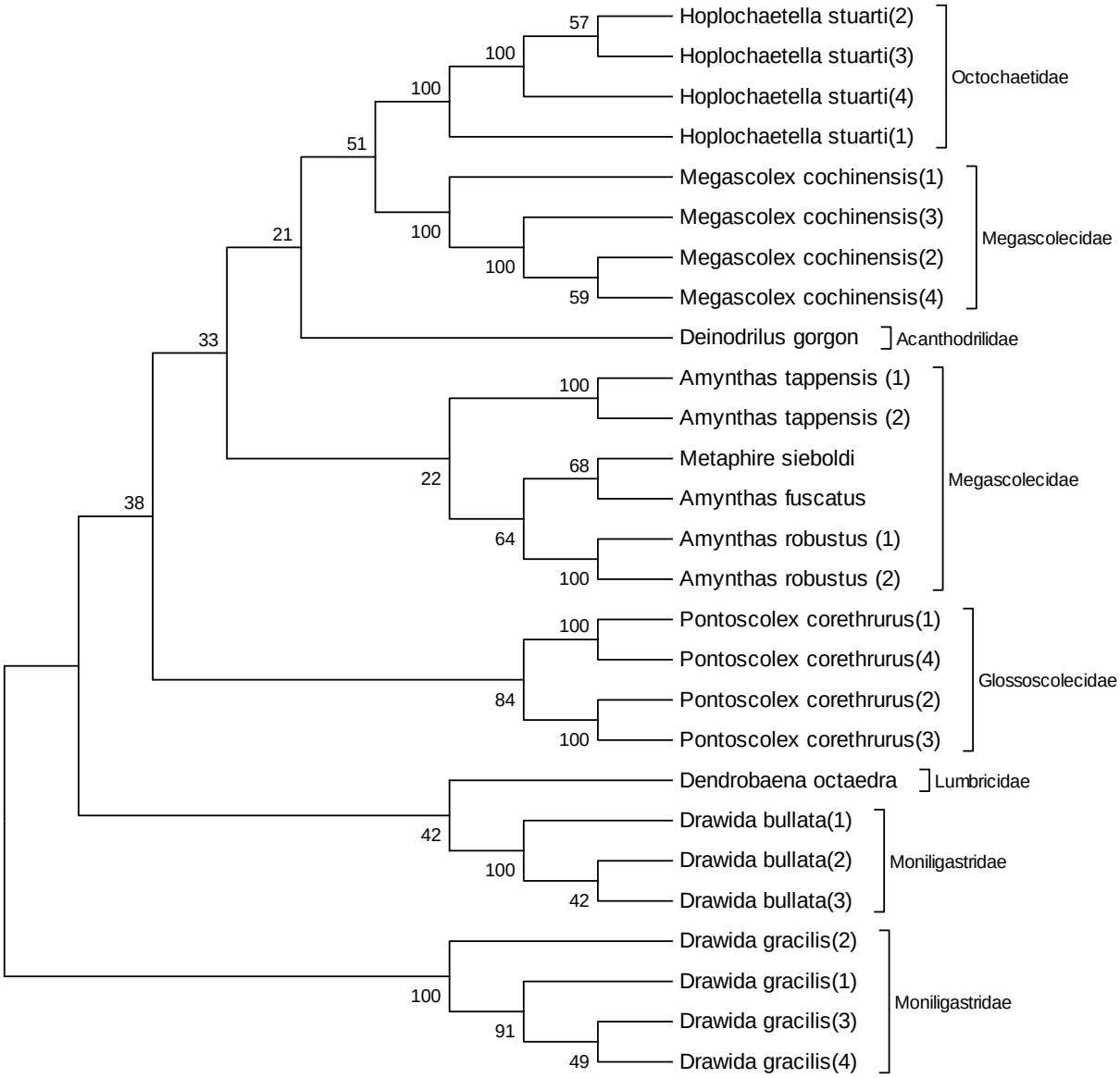

Fig. 3: The evolutionary history was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length = 1.18580199 is shown. The evolutionary distances were computed using the p-distance method and are in the units of the number of base differences per site. Codon positions included were 1st+2nd+3rd+Noncoding. There were a total of 902 positions in the final dataset.

The maximum Log likelihood for this computation was -1576.968. This is commonly observed in earthworms. The clustered sequences show 403 conserved sites, 255 variable sites, 245 parsim-info sites, 10 sigleton sites of 658 bp. From the 658 bp 404 sites zero-fold, 56 sites are two-fold and 35 are four-fold sites. The 326 sites show 100% coverage in 658 bp. The result shows that the distance analysis indicates the overall distance among the five earthworm species as 0.09. Estimates of the Mean Evolutionary Diversity within Subpopulations show 0.01, 0.01 and 0.04 between the *D. gracilis* and *H. stuarti*, *M. cochinensis* and *P. corethrurus* respectively.

The number of transitional differences per site from mean diversity calculations for the entire population is shown as 0.09. The number of transitional differences per site from mean interpopulational diversity calculations is shown as 0.05, 0.06, 0.07 and 0.06 between the *D. gracilis* and *H. stuarti*, *M. cochinensis*, *D. bullata* and *P. corethrurus* respectively. The number of transitional differences per site from estimation of coefficient of evolutionary differentiation is shown as 0.84, 0.87, 1.00 and 0.62 between the *D. gracilis* and *H. stuarti*, *M. cochinensis*, *D. bullata* and *P. corethrurus* respectively.

Discussion

In this present study, the earthworm species were collected from Lateritic Semi Evergreen Forests of Kolli hills, Tamil Nadu, India, and the COI gene sequence was used to identify from these earthworm species. The maximum variations may occur in the sequences between two species and with the same species also, in our study there were no much

variations between the same species within the group and between the allied species of other earthworms groups. Huang et al[16] found that the dichotomy in divergences allowed us to successfully conduct identification tests using the neighbor-joining tree and should enable the reliable delineation of the 86 tested specimens. That is, for 86 specimens, 100% of the COI identifications agreed with the morphological identifications provided by expert taxonomists. In our study, the taxonomy of the earthworm specie *D. gracilis* and *D. bullata* containing external and internal characters is same except the structure of gizzard; in *D. gracilis* four segments of gizzard are present but in *D. bullata* five segments of gizzard are present, the COI sequence is also arranged very closer with one another group and appears as different dichotomy.

M. cochinensis contains the following important taxonomical characters, 224 segments, Dorsal pores from v/vi, Setae perichaetine arrangement, Clitellum xiv- xvii, Male pores as oblique wavy slits, Spermathecal pores in vii/viii and viii/ix, Gizzard large and barrel shaped in v, oesophagus swollen and vascular in xii-xiv. Intestine begins in xix. Huge variations in taxonomic characters with the genus *Drawida* are same like as they formed grouping with huge distance in all trees. This result also confirmed that the COI sequence also identified earthworm species up to species level.

Previously Chih Han et al[7] reported the topology of the NJ tree inferred from the whole dataset clearly illustrating the very strong signal of COI at the species level in earthworms. Traditionally, morphological characters have been used for inferring phylogenies. Zuckerkandl and Paulins's[37] pioneering study showed that molecular sequences provide sets of characters that can carry a large amount of information. *D. gracilis* individuals were highly similar with close values within the same species in estimation of evolutionary divergence between the sequences of the earthworm species collected from our sampling sites. Sequences of the remaining earthworms contain some difference but not much, these differences may occur due to their environmental factors like temperature, soil moisture and their co-factors.

Hebert et al[13] found that over 98% of animal species show greater than 2% divergence and suggested that this was the threshold for spider identification. They proposed a standard sequence threshold of 10, the mean intraspecific variation for the group.[14] In our study, the average intraspecific corrected distance was 9%. This suggests that a sequence-corrected divergence greater than 15% can reliably distinguish recognized species of earthworms. The clusters observed were all monophyletic, strongly supported and deeply divergent. The distribution of intra- and interspecific distances calculated on adult specimens is much like the pattern already documented in Taiwanese earthworms[6] and thus ensuring the efficiency of DNA barcodes as a tag for species discrimination.[24]

Conclusion

We performed a systematic assessment of DNA Barcoding for earthworm species. Therefore, the idea of molecular barcoding for taxonomic purposes is already a reality.[4] Descriptions of new 'species' are being published with a DNA sequence attached to the primary nomenclatorial act[29] and this should be actively encouraged. Taxonomists preparing new taxa for publication should be welcome by DNA-savvy biodiversity laboratories that should be able to provide expertise at minimal cost.

Methods for rapid sequence acquisition for minimal cost are already in existence at genome sequencing centers and are easily adapted for taxonomy of earthworms. Therefore, it is concluded that DNA Barcoding of COI gene of *D. gracilis, D. bullata, H. stuarti, M. cochinensis* and *P. corethrurus* may be used for the identification of these species reported from any part of the world through BLAST analysis if the identical sequences are submitted to any one of the public database in future.

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