



## Oligochaete taxonomy – The rise of earthworm DNA barcode in India

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Oligochaeta is a class of segmented worms under the phylum Annelida that are characterised by the presence of tiny setae in each body segment. Earthworms are the main members, consisting of approximately 6200 species. Their ecological importance is well known as they are the major soil macro-fauna; Aristotle had named them as “the intestines of soil”. Classification of earthworms is a controversial issue since the introduction of modern taxonomical system on earthworm by Michaelsen in 1921. This is mainly because conventional identification using morphological and anatomical characters are complicated and confusing. The key diagnostic features such as the position and structure of the reproductive organs, clitellum and the associated tubercular pubertatis are not always reliable, particularly in different developmental stages, especially when the available specimens are the juveniles. DNA barcoding has offered a potential solution, even at the levels of identifying the juveniles or cocoons. Several genes including mitochondrial cytochrome-c oxidase I, 16S, 18S and 28S ribosomal RNAs, and protein-coding histone H3 genes have been introduced in the taxonomy and phylogeny of earthworm. It is anticipated that DNA barcoding will help conflicting taxonomy and further exploration of species diversity in India.

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### Introduction

A class of annelids with segmented body that possess a simple tiny setae projecting from each segment such as earthworms are termed as Oligochaeta. Aquatic and terrestrial worms belong to Oligochaeta, and majority of the oligochaete taxa are earthworms. Based on ecological and taxonomical perspectives, earthworms are generally considered as a relatively well known group of soil invertebrates which constitutes the

major soil macro-fauna. They drew scientific attention due to their ecological importance as major regulators of important soil processes, or because of their economic importance in agroecosystems.<sup>1,2</sup>

Darwin claimed that no living creatures had such a profound impact in the history of the world as has the earthworm.<sup>3</sup> Being well known organisms during the ancient Greek and Egyptian, as they were able to dig and move in the soil while digesting it, Aristotle called them “the intestines of soil”.

## Historical Record

From a taxonomical point of view, earthworms were mixed with all other worms and it is only in late 18<sup>th</sup> century and the classifications by Lamarck separate annelids (segmented worms) from other worms.<sup>4</sup> To date earthworm diversity described worldwide comprises of approximately 6200 earthworm species<sup>5</sup>, the first being *Lumbricus terrestris* by Carolus Linnaeus in 1758.<sup>6</sup>

A brief history of the oligochaete taxonomy was presented by Csuzdi and Szilávecz<sup>6</sup>, and according to them, there were only 70 new species descriptions published in the first century of earthworm taxonomy (1758–1865), the situation significantly improved by the 1860s when a new generation of zoologists began exploring this important soil engineer worms. Research by Johan Gustaf Hjalmar Kinberg (1820–1908), Edmond Perrier (1844–1921), Gustav Eisen (1847–1940), Rutgerus Horst (1849–1930), László Örley (1856–1887), Daniel Rosa (1857–1944) and Frank Evers Beddard (1858–1925) greatly enriched the knowledge of earthworm systematics.<sup>6</sup> Johann Wilhelm Michaelsen (1860–1937), Sir Walter Baldwin Spencer (1860–1929), Sir William Blaxland Benham (1860–1950) and Luigi Cognetti De Martiis (1878–1931) make fundamental contributions to earthworm taxonomy.<sup>6</sup> Csuzdi and Szilávecz (2016) called the period 1886–1905 as “the golden age” of earthworm taxonomy because of the enormous and significant contributions made by Örley (1885), Benham (1886), Beddard (1895), Rosa (1893, 1895) and Michaelsen (1900, 1903).<sup>6</sup>

After the outbreak of the World War I, Stephenson had contributed immense taxonomic information of Oligochaeta in his books - *The Fauna of British India* (1923) and *The Oligochaeta* (1930). The majority of earthworm systematics available was perhaps the outcome of his work. Gordon Enoch Gates (1897–1987), Lev Černosvitov (1902–1945) and Grace Evelyn Pickford (1902–1986) added Michaelsen and Stephenson's taxonomic work.<sup>6</sup> Later, after the World War II, the dwindling oligochaete taxonomic interest was revived by Kenneth Ernest

Lee (1927–2007), Pietro Omodeo (1919–) and Barrie Gillean Molyneux Jamieson (1934–). Danuta Jadwiga Plisko (1927–), Reginald William Sims (1926–2012), Marcel Bouché and Gilberto Righi (1937–1999), András Zicsi (1928–2015) and others had performed their earthworm identification works.<sup>6</sup> Many other workers from different parts of the globe had contributed a lot since last few decades. In Indian subcontinent, J.M. Julka is serving as the sole earthworm taxonomists since 1980's till date. In addition to this, R. Paliwal and other did some works in the field of earthworm's morpho-anatomical taxonomy mainly based on monographs. All these highly skilled efforts are based on morphological and anatomical investigation, which is known as “conventional method of identification”.

## The Taxonomic Impediment

Regarding the classification of earthworms, there is controversy over the view of different scientists. Some scientists have developed their own classification schemes which have been further revised and developed over years. Basis of modern taxonomical system on earthworm was formed by Michaelsen (1921).<sup>6</sup> Later, Stephenson (1930) simplified Michaelsen's classification of earthworms into fourteen families. The classification of the family megascolecidae has been more controversial as Omodeo (1958), Gates (1959) and Lee (1959) independently proposed their own classification. Later Sims<sup>7</sup> investigated with computer techniques, and follow Gates classification of megascolecidae. In order to study the biological as well as the ecological process of earthworm, a correct species diagnosis is vital.

Traditional method of identification deals with morphological as well as anatomical characters, such as the position of spermathecal pore, first dorsal pore, arrangement of setae, shape and segments covered by clitellum, position and shape of female and male pore, penial setae and genital markings are of taxonomic importance in its morphology. The chief anatomical features of systematic importance includes the gizzard, calciferous glands, the seg-

ment in which the intestine begins, presence and position of intestinal caeca, typhlosole, the details of vascular systems, nephridia, sex organs like testes, spermathecae, seminal vesicles, prostate glands, ovary, oviduct and ovisac. All these characters were considered while identifying earthworm species. But still the key characters considered for correct taxonomic identification differs from species to species.

However, conventional identification using morphological and anatomical characters are complicated as this method requires minute and detail examinations of external morphology as well as internal anatomy of adults, which necessitates the engagement of expert taxonomists. This method is time consuming and very laborious work, thus it is considered cumbersome. Furthermore, identification is limited to sexually mature specimen as the key character in species diagnosis involves the position and structure of the reproductive organs, clitellum and the associated tubercular pubertatis.<sup>8,9</sup> As a result, identification of juveniles of closely related species (e.g. members of the genus *Lumbricus*) is not feasible in most cases such that soil invertebrate taxonomic studies are greatly hampered as only generic identifications for juveniles are available for species richness evaluation.<sup>10</sup> In addition, diverge taxonomic system can results between researchers where they use different criteria or sections of the earthworm for identification since, the degree of variability of many morphological and anatomical characteristics of earthworms can differ and features can overlap between taxa.<sup>11</sup> For instance since 1913 till date, a good number of re-description of earthworm species have been recorded for a number of taxa.<sup>12-25</sup>

Many described species of earthworm comprising several distinct genetic lineages were hypothesized to represent cryptic species, the distinction of which is difficult if not impossible on a morphological basis has been revealed by the use of molecular approaches in biodiversity research, thus, suggesting that traditional morphological identification might be inaccurate. Taking into consideration for cryptic diversity has profound implications for earthworm taxon-

omy. For instance, Bouche and Beugnot (1972)<sup>26</sup> described the two morphs of *Lumbricus terrestris* which later was observed that these two morphs are in fact two distinct species which were both formally named but considered as synonyms for a long period, the large body-sized *L. terrestris* sensu stricto and the smaller *L. herculeus* (Savigny).<sup>27</sup> The existence of at least five different cryptic species in Hormogastridae *Hormogaster elisae* was also reported.<sup>28</sup> Extensive analysis of 457 sequences, representing 49 genera within 8 families, a very high level of potential cryptic diversity, with about 1 for 6 identified species having one or more divisions exceeding 10% divergence was observed by Rougerie *et al.*<sup>29</sup>. It is thus obvious that there are some limitations with conventional morpho-anatomical identification, particularly between intra-specific variations.

### Modern Molecular Approach

The modern molecular approach- DNA based species identification can provide solution for such taxonomic impediment by providing a highly reliable automated identification tool usable for any life stages and on any kind of tissue fragments. Indeed, reliable identifications of juvenile specimens<sup>29-31</sup> which conventional morpho-anatomical identification cannot accomplish.

DNA barcoding, a means of species identification is a technique in biodiversity research<sup>32</sup> which uses information *i.e.*, a standardized region of DNA common across all taxa, by DNA sequencing under universal condition. This gene region constitutes a small part (<1000 bp) of the genome and can be easily obtained. These features leads to standardization across species and laboratories, thus providing a platform for global exchange of homologous data and capitalizing on the efforts of others to build a more flexible system.<sup>33</sup> For DNA barcoding of animals, the Mitochondrial DNA (mtDNA) which uses 658 bp fragments of the mitochondrial cytochrome-c oxidase I CO1 gene can be used as a standard DNA tag to identify individuals belonging to the same species, as well as to distinguish between

individuals from different species and identification for phylogenetic studies of the animal kingdom.

This has turned out to be exceedingly useful in tracing evolutionary history<sup>34</sup> as it is present in all eukaryotic organisms, and are maternally inherited, and most of the nucleotide substitutions occur at neutral sites, evolves much more rapidly as compared to nuclear DNA thereby resulting in the accumulation of differences between closely related species.<sup>35-37</sup> Nuclear and mitochondrial genomes exhibit different patterns of inheritance.<sup>38</sup>

In a more advance level, COX 1 sequence data have been used to study the intra and inter-phylogenetic relationships. Relative homogeneity is maintained by concerted evolution, where mutations rapidly spread to all members of the gene family even if there are arrays located on different chromosomes.<sup>39-41</sup> The DNA barcodes based species or lineage discrimination is congruent in many cases with those obtained with other molecular markers. For instance, result obtained from AFLP is in congruent with COI for Great Britain Lumbricidae, microsatellites support the integrity of most COI lineages described by King *et al.*<sup>42</sup> for *Allolobophora chlorotica* (Savigny).<sup>43</sup> A high congruence between COI lineages and those obtained with 16S t-RNAs and 28S within the morphologically similar species *Hormogaster elisae* (Álvarez) was also documented by Novo *et al.*<sup>28</sup> Tree-based taxon clustering as well as statistical taxon separation analysis indicates that molecular evidence does coincide with morphological hypotheses.<sup>44</sup> Hence, species identification based on DNA sequence analysis proved to be feasible for the analysed taxa.

### DNA Barcoding for Earthworm Species Characterization

Use of DNA barcoding for identification of earthworm species is improving our capability in describing juveniles or cocoons into oligochaete biodiversity study. Similarly, molecular identification will enable the use of damaged or broken specimens for identification which otherwise

present inadequate observable characters for conventional identification. Relatively few molecular analysis tools have been developed for earthworms, despite the economic and ecological importance of these soil ecosystem engineers.<sup>45</sup> Several genes such as mitochondrial cytochrome-c oxidase I, COI<sup>10,11,46-57</sup>; 18S ribosomal RNA<sup>11,48,57-59</sup>; 28S ribosomal RNA<sup>51,54,56,58</sup>; 16S ribosomal DNA<sup>11,48,49,51,54-57,59</sup> and protein-coding histone H3 genes<sup>54,56</sup> have been recently used for studying the taxonomy and phylogeny of earthworm species. Loongyai *et al.*<sup>60</sup> have obtained partial sequences for ribosomal 16S rDNA and subunit one for mitochondrial cytochrome c oxidase (COI) of four earthworm species and reported that result of sequence analysis combined with taxonomic characters could distinguish the different species of earthworm. First overview of DNA barcoding approach for discrimination, identification of new taxa, and species identification of juveniles and detection of cryptic diversity for earthworm species was provided by Rougerie *et al.*<sup>29</sup> A number of studies have been published which highlight the potential of DNA barcoding in opening new avenues in the field of earthworm ecology, phylogeography and population genetics.<sup>47,61,62</sup>

As compared to morphological taxonomy, DNA barcoding provides more rapid progress on species identification.<sup>63</sup> It can sustain decisions taken for nomenclatural acts (alpha taxonomy) including descriptions of new taxa, synonymies or rehabilitation of synonymized species names. The method, alone or as a complement with new generation environmental sequencing, also represents a promising tool for biodiversity assessment from local to global scales, or for studying the biogeography and population genetics of specific species or lineages.<sup>64</sup> Adopting DNA barcoding is enhancing the accuracy of earthworm studies and more generally benefit the community of soil biologists. Thus, recently many papers has been published and new earthworm species have been described with the help of DNA barcoding.<sup>52-58,65-73</sup>

Once DNA barcodes have unveiled putative cases of cryptic diversity, morphological features needs to be re-evaluated for species discrimina-

tion.<sup>27</sup> Recent studies suggest that characters which have long been considered as uninformative for species discrimination and description might be far more informative than previously assumed due to DNA barcoding.<sup>46,62,74,75</sup> There are reports that taxonomists scrutinize the DNA barcoding analyses results, using them when relevant within the traditional workflow of new species descriptions and taxonomic revisions<sup>76–80</sup> thus, enabling reliable identification.

DNA barcoding have been supported by different national and international initiatives, of which the International Barcode of Life project (iBOL, <http://ibol.org/>) is the most determined one in term of investment. The sequences generated are collectively compiled in the Barcode of Life Data System (BOLD, <http://www.barcodinglife.org>), a central integrative bioinformatics platform, which serves as a scientific workbench supporting all phases of the analytical pathway from specimen collection to tightly validated barcode library.<sup>81</sup> A specific “Barcoding Earthworms” campaign (EarthwormBOL) which assembles libraries of DNA barcodes from samples collected all over the world was launched. To date, the earthworm reference library contains more than 18000 sequences for over 1200 species from more than 49 different countries; these numbers are expected to rise quickly within the next few years.<sup>82</sup> Barcode data can also be used as a support or additional evidence in the description of new species to avoid taxonomic confusion.<sup>62</sup>

### Earthworm Species Diversity and DNA Barcoding - Indian Scenario

India is one of the important mega-biodiverse countries. It harbours about 11 % of global earthworm diversity which includes about 509 species placed in 10 families and 69 genera.<sup>83</sup> No information was available from India on molecular characterization and diversity of earthworm species till 2008.<sup>84</sup> However, Meenatchi<sup>85</sup>, Giraddi *et al.*<sup>86</sup> and Meenatchi *et al.*<sup>87</sup> used RAPD-PCR method for the first time to assess the genetic diversity of Indian earthworms. Other preliminary works includes Kumar *et al.*<sup>65</sup>

Mathur *et al.*<sup>88</sup> Sharma *et al.*<sup>89</sup> and Biruntha *et al.*<sup>90</sup> Various molecular markers are being used such as Random Amplified Polymorphic DNA (RAPD), Restriction Fragment Length Polymorphism (RFLP) and Simple Sequence Repeat (SSR)<sup>89,91,92</sup> which have profound use in genetic diversity estimation.<sup>93</sup> Rao *et al.*<sup>58</sup> has performed phylogenetic analysis and multiple sequence alignment of *Eudrilus eugeniae* 18S rRNA and observed a pattern of variations among different species. Kushwaha *et al.*<sup>94</sup> published their modified protocol for earthworm DNA extraction. Jaya *et al.*<sup>66</sup> have sequenced the mitochondrial CO1 gene of six species of earthworms and observed that the cytochrome-c oxidase I (CO1) exhibited a unique barcode to a particular species. Earthworm DNA barcoding work in India is at its initial stage, such that very little information is available. Recently, a project on diversity studies of Indian earthworms using DNA barcodes has been initiated by Shweta Yadav. A total of 801 of Indian earthworm DNA sequence have been made available in the Boldsystem such as Acanthodrilidae 4, Almidae 3, Eudrilidae 22, Hormogastriidae 7, Lumbricidae 6, Megascolecidae 426, Moniligastridae 122, Octochaetidae 199, Rhinodrilidae 6.<sup>82</sup> This represents only a small portion of Indian earthworms and therefore further challenges remain open for the enthusiast biologists to undertake research in the field of molecular approach, the DNA barcoding for earthworm species identification. The most recent description (2017) of new earthworm species from India, the discovery of two new Moniligastrids species viz. *Drawida polydiverticulata* sp. nov. and *Drawida thomasi* sp. nov. are not provided with molecular data.<sup>95</sup>

Further scientific investigations on earthworm diversity and alpha taxonomy are, therefore, required and it is imperative to make an attempt conducting an investigation on earthworm species and genetic diversity available in different unexplored soil habitat conditions of India, particularly of north-east India as it lies within the Himalaya and Indo-Myanmar biodiversity hotspots. The most reliable identification of earthworm taxa is now based on the combination of the conventional morpho-anatomical

characters and molecular characters (DNA barcode), therefore further exploration of species diversity and genetic diversity as well as phylogenetic analysis of earthworms Indo-Myanmar and Indo-China of northeast India has been initiated by the authors. This will enrich the knowledge of earthworm diversity of India and the world by providing fundamental information on biodiversity and molecular phylogenetic data of earthworms of north east India.

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