Disentangling earthworm taxonomic stumbling blocks using molecular markers

Azhar Rashid Lone1, Samrendra Singh Thakur2, Nalini Tiwari3, Olosola B. Sokefun4 & Shweta Yadav5

1,3,5 Department of Zoology, Dr. Harisingh Gour Vishwavidyalaya (A Central University) Sagar, Madhya Pradesh 470003, India.
2 Department of Biotechnology Dr. Harisingh Gour Vishwavidyalaya (A Central University) Sagar, Madhya Pradesh 470003, India.
4 Department of Zoology and Environmental Biology, Faculty of Science, Lagos State University, Ojo, 102101, Lagos, Nigeria.
1 rashidazhar444@gmail.com, 2 samrendra.thakur01@gmail.com, 3 n.tiwari2987@gmail.com, 4 osokefun@gmail.com, 5 kmshweta@gmail.com (corresponding author)

Abstract: Taxonomic classification of earthworms based on anatomical features has created several challenges for systematics and population genetics. This study examines the application of molecular markers, in particular mitochondrial cytochrome (COI), to facilitate discrimination of closely related earthworm species. Molecular markers have also provided insights into population genetics by aiding assessment of genetic diversity, lineage sorting, and genealogical distributions of populations for several species. Phylogeography—a study that evaluates the geographical distribution of these genealogical lineages and the role of historical processes in shaping their distribution—has also provided insights into ecology and biodiversity. Such studies are also essential to understand the distribution patterns of invasive earthworm species that have been introduced in non-native ecosystems globally. The negative consequences of these invasions on native species include competition for food resources and altered ecosystems. We anticipate that molecular markers such as COI and DNA barcoding offer potential solutions to disentangling taxonomic impediments in earthworms and advancing their systematics and population genetics.

Keywords: Annelida, COI, cryptic species, genetic diversity, Invasive species, Oligochaeta, phylogeography, phylogeny.
INTRODUCTION

The terrestrial Oligochaeta include annulated worms known as earthworms or megadrodes, a group of invertebrate animals dispersed all over the world and having a paramount role in the development of burgeoning soil and its fertility (Lavelle et al. 1999; Edwards 2004). At present, the earthworms are investigated all over the world by approximately 300 specialists, most of them aiming at their ecology and role in terrestrial ecosystems. A few tens of earthworm scientists are considered to have expertise in Oligochaeta worm taxonomy and phylogeny. Terrestrial Oligochaeta has a relatively short and somewhat simple history. Started with the work of Savigny (1826), the study of earthworms gradually involved more specialists and consequently became more complicated as new characters and taxa were described. The contemporary terrestrial oligochaete taxonomy is considered as being rooted in the classical works of Rosa (1888–1944) and Michaelsen (1830–1930). Later Pop, Omodeo, Perrel, Zici, and Bouche contributed substantially to the knowledge of earthworm (especially Lumbricidae) taxonomy and phylogeny. The studies of earthworms got rapid worldwide development in the second half of the 20th century with the development of soil science and soil zoology. Scientists all over the world were invigorated to study earthworms by the general acceptance of the idea of the soil, as indispensable for agriculture and must be carefully managed to avoid its irreversible deterioration. At that time, soil-inhabiting animals began to be looked as ‘main soil builders’ not only by a few zoologists, but by a large circle of specialists interested in improvement and conservancy of soil productivity. Only in a few decades, the main interest of specialists targeted more and more at the ecological aspects of soil inhabiting animals. More applicable fields were separated from the theoretical aspects by the processing of organic materials by earthworms. It also proved to merit protection from the negative effects of pesticides and even some fertilizers. A large section of scientists turned their interest to the study of earthworms. But at the same time, a classical field of earthworm taxonomy and phylogeny didn’t magnify equally. The novelty and ecological approaches of the animal overlapped their basic studies. The majority of active scientists turned their interest to the ecology and application part of earthworms and the earthworm taxonomy was somewhat neglected or even considered to be outdated. Nevertheless, due to large-scale faunistic investigations, promoted by the biodiversity and ecosystem structure investigations, a lot of unknown taxa were found and described. The scarcity of skilled taxonomists led to the inflation of improperly described earthworm taxa and the appearance of parallel classification. Ecologists were firstly affected, but even specialists hardly succeeded to extricate the entangled stumbling block of earthworm taxonomy. It became obvious to develop a technology to resolve taxonomic impediments with the use of molecular tools while the traditional taxonomy and modern molecular taxonomy have contributed equally to the advancement of earthworm taxonomy.

Traditionally, earthworms are characterized based on classical approaches like morphological investigations of the external body and anatomy-based dissections which take the advantage of limited taxonomic parameters like the structure of prostate, seminal vesicles, spermatheca, and calciferous glands (Lalthanzara et al. 2018). Moreover, due to simplicity of their structural organization, several diagnostic characteristics in earthworms are inconsistent and overlaps beyond taxon (Perez-Losada et al. 2009), their characterization requires experts which unfortunately are splurging. The shortage of discriminatory characters in earthworms was first divulged by Michaelsen (1900) and consequently defined these animals as ‘sine systemate chaos’. Thus in all the domains of earthworm research, the existence of these taxonomic impediments is responsible for major prejudices. The use of a molecular approach may be a potential resolution to tackle the stumbling block of earthworm taxonomy. The use of a standard mitochondrial genetic marker often termed DNA barcoding has been, nowadays, considered as a reliable approach used in biodiversity studies as well as in species identification (Hebert et al. 2003, 2004). Chang et al. (2008) and Rougerie et al. (2009) have given voice to DNA barcoding as a potential solution to disentangle taxonomic impediments.

The study reviewed the prospective of molecular approaches including short sequences of the mitochondrial genome, in particular, the COI and its preponderance in resolving the stumbling block of earthworm taxonomy. The present study accentuates the contribution of this gene marker in deciphering taxonomic impediments primarily identification of species, phylogeny re-constructions, intraspecific variations; genetic structure, cryptic species, lineage sorting, and finally its role in the assessment of invasive species with phyllogeographic tagging (Figure 1).
DNA Barcoding and Clitellate species identification

Before DNA barcoding earthworm taxonomy relied on the specific morpho-anatomical features, however, most of these features often overlap among taxa and it became more inadequate when recently divergent species or species complexes were entertained (Chang & James 2011). Although, the allozymes, RAPD, RFLP, and SSR techniques in the mid-19th century reflected the notion that certain earthworm species could be segregated. Nonetheless, due to their certain limitations including dominance and less reproducibility, the focus was given to the use of various gene markers to gain a better understanding of earthworm taxonomy (Kumari & Thakur 2014). DNA barcode occupies 658 bp of the mitochondrial genome for the recognition of animal species (Hebert et al. 2003). This method has diverse advantages; firstly, it is a rapid and cheaper technique in the case of massive samples for accurate identification. Secondly, it is reproducible and testable since it always keeps the record between any barcode and its voucher specimen. Above all, it could be applicable for tissues and applied to any life stages whether cocoons or a juvenile of any animal species as well as it is accessible everywhere around the globe (Rougerie et al. 2009). DNA barcoding has the potential for earthworm research in taxonomy and ecology (Decaëns et al. 2013). Moreover, in eco-toxicological studies, it is very essential to identify accurate model organisms for inferring toxicity of several compounds, as it is evident that many closely related species can react to the same toxicant differently. Otomo et al. (2009) highlighted the importance of DNA barcoding for the identification of earthworm species used in ecotoxicological tests and concluded that reliable identification is very crucial since it prevents various discrepancies when comparative studies are done involving different test species. Similarly, to evaluate the practicability and consistency of DNA barcoding, an international ring test was organized by Römcke et al. (2016) who assessed the genetic differentiation of two ecotoxicological earthworms, viz., Eisenia fetida and Eisenia andrei. These investigations have not only assessed the potential of DNA barcoding in taxon identification but specify that it could be the only way to measure an accurate level of biodiversity (Proudlove & Wood 2003). The study of Richard et al. (2010) shows the potential of DNA barcoding can be applied to identify juvenile earthworm species in soils when reference DNA barcode library is available and thus highlighted that the bias in juvenile collection and identification could be highly reduced in earthworm biodiversity assessments. Moreover, many earthworm taxonomists emphasized that integrating morpho-anatomical features with barcoding data provide more contrasting conclusions. These integrative approaches were utilized to discriminate among species and taxa that are new to science (Shekhovtsov et al. 2014; Jerathitikul et al. 2017; Lone et al. 2020). Furthermore, compared to morpho-anatomical features that require exhaustive work, species discrimination using DNA barcoding is relatively rapid and identification measure is progressed (Gregory 2005). These in turn have addressed certain issues, including rehabilitations, synonymies, and description of new taxa. Thus it sustains the decisions of nomenclature experts and thus primarily contributes to biodiversity assessments from local to global scales. Therefore, adopting DNA barcoding has enhanced the accuracy of earthworm studies and in particular, greatly benefited the community of soil biologists in the description of many novel species over the past few years (Blakemore 2013; Zhao et al. 2015; Aspe et al. 2016; Cszudi et al. 2017; Seesamut et al. 2018; Lone et al. 2020); see Table 1 for more details. Furthermore, DNA barcoding has also shown its congruent results with other nuclear and mitochondrial genes (Pop et al. 2007; King et al. 2008) and many such papers are published in peer-reviewed journals. Furthermore, the nuclear and mitochondrial genes greatly differ in their divergence rates at different taxonomical levels. In many studies, it has been inferred that the mitochondrial gene particularly COI has the highest sequence divergences than other mitochondrial (12S, 16S) and nuclear genes (18S and 28S) (Chang & James 2011). This indicates that at the species levels or intraspecific variations, species could be better studied when the fast-evolving genes like COI are considered. However, at higher taxonomical levels (within a genus or interfamilial) COI has a relatively weak signal than other slow evolving genes (18S, 28S) (Chang & James 2011) and should be used at the species level or within genus if the genus is not too diverse. Thus, COI has been one of the most influential gene markers which have strongly revolutionized earthworm taxonomy by avoiding taxonomic confusions and providing additional evidence for discrimination of taxa over the past few years.

Role in Phylogeny reconstructions

Dobzhansky (1973) stated that in biology, nothing makes sense without the consideration of evolution. Since species undergo evolutionary changes, the relationship of these changes at all levels provides perception in the phylogenies of diverse species. The collaboration of morphological and molecular methods
has shaped significant progress in understanding the phylogeny of most major invertebrate groups (O’Grady & DeSalle 2018). However, this is partially true for the earthworms which have not been resolved, although many attempts were made. About 100 years ago and throughout the greater part of the 20th century oligochaetes, sensu stricto were classified into two main groups: Megadrila and Microdrili. The classification was based largely on two parameters; size and habitat preferences. The larger group that is confined to soils was termed Megadrili and the smaller group that is mostly restricted to water was called Microdrili (Benham 1890). Later Beddard (1895) compiled the basic structure laid out by Benham, however, redefined Microdrili by adding the family Naidomorpha’ (presently called Naididae) - a group that Benham considered as a subclass distinct from the rest of the oligochaetes. Following cladistic analysis and reclassification of Oligochaeta, Jamieson (1988) anticipated a new name for the Megadrili group, Metagynophora, based on the inferred loss of ovaries located anteriorly. He also proposed Crassicilitellata a less inclusive taxon for about 3,000 earthworms, containing multi-layered clitellum (composed of several epidermal cell layers). Whilst, other oligochaetes app.

120 Metagynophora species that mostly belonged to the family Alluroidiidae and Moniligasteridae, outside Crassicilitellata, contained single-layered clitellum. The molecular phylogenetic analysis although started in the 1990s however, it was not until Siddall et al. (2001) for the first time focused on the phylogenetic study of leeches and their relatives that also included earthworms. Later, Jamieson et al. (2002) published their work on the phylogenetic study of earthworms and revealed monophyly of the Megascolecidae family based on 12S, 18S, and 16S data, besides it supported the clade Crassiclitellata (Jamieson 1988). Subsequently, many papers were published on the phylogeny of earthworms (Table 2). Moreover, to construct a phylogeny in earthworms, the selection of accurate markers would be essential. **COI** is preferred due to its simplicity of primer design and range of its phylogenetic signal (Hebert et al. 2003), rapid evolution to discriminate at the species level (Wishart & Hughes 2003), and to provide informative features (Siddall et al. 2001; Pop et al. 2003; Heethoff et al. 2004; Chang & Chen 2005; Pérez-Losada et al. 2005; Chang et al. 2007, 2008; Huang et al. 2007; King et al. 2008). Although many other genes are taken into consideration for the construction of phylogeny in

| Marker(s) | Main focus | Region(s) | Reference |
|-----------|------------|-----------|-----------|
| COI       | New species (*Pontodrilus longissimus*) description | Thailand and Peninsular Malaysia | Seesamut et al. 2018 |
| COI       | Description of new species *Eisenia nordensioidi mongal* and *Eisenia nordensioidi anon* | Mongolia | Blakemore 2013 |
| COI/ITS   | Aquatic oligochaetes identification | Switzerland | Vivien et al. 2015 |
| COI/morphology | New taxa identification | Kamchatka | Shekhovtsov et al. 2014 |
| COI/16S/18S/28S/H3/H4/tRNAs | Description of new species *Eiseniona gerardoi* within Lumbricidae | Extremadura, Spain | Cosin et al. 2014 |
| 16S/28S/COI/H3/ tRNAs | Description of new species *Hormogaster joseantoni* | Teruel Aragon ranges, Aragon, Spain | Marchán et al. 2014 |
| COI       | DNA barcoding of *Kanuchuria* species | Megalaya, India | Lone et al. 2020 |
| COI       | DNA barcoding of *Eutyphoeus* species | Mioram, India | Thakur et al. 2020 |
| COI       | DNA barcoding of earthworms species | Madhya Pradesh, India | Tiwari et al. 2020 |
| COI       | DNA barcoding | Thailand | Jeratthitikul et al. 2017 |
| COI/16S   | DNA barcoding and phylogeny in genus *Glyphidrilus* | Thailand | Jirapratrasilp et al. 2016 |
| COI       | DNA barcoding | Arunachal Pradesh, India | Lalthanza et al. 2020 |
| COI       | DNA barcoding in *Amynthas* genus | Northeastern India | Valenýurelai et al. 2020 |
| COI       | DNA barcoding | Uruguay | Escudero et al. 2019 |
| COI       | Description of new taxa | Taiwanese montane | Chang et al. 2007 |
| COI       | DNA barcoding | China | Huang et al. 2007 |
| COI       | DNA barcoding | Taiwan | Chang et al. 2009 |
| COI       | DNA barcoding | Canadian Centre for DNA Barcoding (CCDB) | Rougerie et al. 2009 |
| COI       | DNA barcode for juvenile ID | Haute-Normandie, France | Richard et al. 2010 |
earthworms (see Table 2), COI is generally engaged for its rapid divergence and fast-evolving features that aid in a better understanding of evolution and phylogeny reconstructions. Irrespective of being a vital role and promising idea that DNA barcoding has given to the molecular phylogenetics, the ongoing debates on earthworm systematics still face many key challenges that need to be addressed (Chang & James 2011). Perhaps, these overwhelming challenges are not only confined to earthworms but also the whole Annelida. As McHugh (2001) stated that the poor resolution at higher levels in Clitellata is due to radiation or rapid divergence of annelid phylogeny and Martin et al. (2000) stated the same reasons for Clitellata phylogeny which was also supported by the investigation of Maekawa et al. (2001) and Su et al. (2001). This demands further research and large datasets to answer the key questions in Clitellata phylogeny. Although the molecular phylogenetic investigations were studied in the family Eudrilidae, Ocnerodrilidae, Lumbricidae, Megascolecidae, and Glossoscolecidae, however, except for the support of the monophyly in Megascolecidae, the support for all the families is weak due to insufficient sampling and taxon bias. Moreover, in the family Lumbricidae, the focus is given to within genus (Aporrectodea/Allolophora, Dendrobaena, and Octodriloides/Octodrilus/Octolasion) which led to restating the polyphyletic nature of Allolophora and synonymizing Octodrilus with Octodriloides, nonetheless, there was no significant progress in phylogenetic revision (Pop & Wink 2004; Pop et al. 2003, 2007, 2008; Cech et al. 2005). Thus we can anticipate that the phylogeny of the oligochaetes Clitellata still encompasses various challenges in the present scenario, and requires further development for in-depth phylogenetic information. Moreover, DNA barcoding has no doubt interpreted many findings either alone or with the combination of other genes however, more data is required to tackle many challenges in phylogenetic studies in Clitellata and lastly the more densely the taxa are sampled, the more defined the phylogenetic estimations will be measured (Erséus 2005).

Table 2. List of some peer reviewed publications in earthworm phylogeny and systematics.

| Marker(s) | Main focus | Region(s)/Platform | Reference |
|-----------|------------|--------------------|-----------|
| COI       | Phylogeny of Eisenia. nordenskioldi | Siberian and Korean | Hong & Csuzdi 2016 |
| COI/16S/18S/28S/H3/H4/tRNAs | Hormogastridae phylogeny | 46 sites in the Iberian Peninsula to Corsica and Sardinia | Novo et al. 2011 |
| COI/COI/12S/16S | Phylogeny reconstruction of Hormogastridae | Mediterranean | Novo et al. 2015a |
| 18S/28S/12S/16S/ND1/COI/COI/tRNAs | Phylogeny of Lumbricidae | Iran | Bozorgi et al. 2019 |
| COI/COI/12S/16S/18S/28S/ND1/tRNAs | Evolution of lumbricids | Europe, USA, Brazil, Africa, UK, China, Israel, Turkey, and Vietnam | Domínguez et al. 2015 |
| 28S/12S/16S/ND1/COI/tRNAs | Lumbricidae phylogeny | Northwestern Spain | Domínguez et al. 2017 |
| COI/16S/ITS2 | Phylogenetic analysis of the Dendrobaena bylbico | Balkans, the Greek islands, Anatolia, Levant and the Carpathian Basin | Szederjesi et al. 2018 |
| COI | Hormogastrid phylogeny | Iberian Peninsula | Novo et al. 2009 |
| COI/28S | Monophyly and phylogeny in Eisenia fetida and Eisenia andrei | Ireland and Spain | Pérez-Losada et al. 2005 |
| 12S/16S/28S/COI/ND1/tRNAs | Phylogenetic relationships of Aporrectodea caliginosa species complex | European earthworms | Pérez-Losada et al. 2009 |
| COI/12S/28S/16S/H3/ITS | Phylogeny of Lumbriculus | North America, Europe, Japan, and China | Liu et al. 2017 |
| COI/16S/H3/ITS2 | Cognettia diversity | Northern Europe | Martinsson & Erséus 2014 |
| COI/COI/COI/Cyts/NDS/ND4/16S/ND1 | Phylogenetic relationships of 15 Pheretima complex | China | Zhang et al. 2016 |
| COI/COI/28S/H3 | Phylogeny of A. caliginosa complex | Europe, UK, USA, Egypt, Australia | Fernández et al. 2012 |
Unveiling cryptic species/species complex/intraspecific divergence in Clitellata

In the biological process, cryptic speciation results in a species group, containing individuals that are morphologically identical to each other however belong to different species (Pérez-Losada et al. 2005). With morpho-anatomical features, most of the cryptic species/species complexes remain unnoticed and it was not until with the availability of DNA sequences there was an increase in the number of cryptic species (Torres-Leguizamon et al. 2014; Marchán et al. 2017). In earthworm taxonomy, the identification of taxa at higher taxonomical levels particularly at genus or interfamilial levels can be studied effectively as there are many taxonomical characteristics that could be applied to assign taxa at family and genus levels (Pérez-Losada et al. 2005). However, when closely related species and species complexes are considered, few morpho-anatomical features are available and it makes taxonomy more complicated when these morpho-anatomical features overlap among them (Lalthanzara et al. 2018). Thus at the species level or when dealing with cryptic species, the taxonomic methods are complicated, exhaustive, labor-consuming, and demands expertise in the field (Lalthanzara et al. 2018; Thakur et al. 2020). Furthermore, due to simple body structures in earthworms, their identification is limited to mature specimens as the key taxonomical features can only be applied to them, leaving juveniles or closely related species unidentified. With DNA barcoding several cryptic species/species complexes are identified in earthworms, most of which are widespread in several families; Lumbricidae (Heithoff et al. 2004; King et al. 2008; Fernández et al. 2011; Shekhovtsov et al. 2013, 2016a), Mediterranean Hormogastridae (Novo et al. 2010, 2011), Megascolecidae (Chang et al. 2008; Buckley et al. 2011), Glossoscolecidae (de Faria et al. 2013) respectively (see Table 3 for more published papers). Moreover, the development of DNA barcoding cryptic species in earthworms has gained pace as more and more data is being added which not only tells us the extensive occurrence of cryptic diversity in earthworms but the action of various ecological processes that has led to these divergences within them. Furthermore, many investigations revealed that several earthworm taxa may contain two to five cryptic lineages with app. 10–20 % of nucleotide substitutions among them (Novo et al. 2009; Buckley et al. 2011; Porco et al. 2013; Fernández et al. 2016). In soil-dwelling invertebrates particularly earthworms the occurrence of these cryptic lineages is common due to allopatric isolation which restricts gene flow between regions of suitable habitat (Hogg et al. 2006) as well as minimizes the change in morphological characters taking place during speciation (Bickford et al. 2007).

In addition to this, the different individuals of a given species are not genetically identical. Their DNA sequences differ to some extent, and these differences form the genetic diversity, known as the intraspecific diversity of a species (Stange et al. 2020). These genomic variations are the basic foundation of biodiversity. It refers to a process by which the characteristics of living organisms change over many generations and addresses how different species are related through the complicated family trees. Understanding diversity at the genomic level including an arrangement in taxonomic standards is, therefore, the most important parameter of biodiversity. The importance of genetic variation in biodiversity evaluation has been well recognized (Des Roches et al. 2018). Nonetheless, such studies cannot be accomplished entirely based on simple morphological examinations of different taxa and therefore demand molecular investigations to provide more tangible understandings of earthworm diversity indices. Moreover, molecular studies, for example, systematic studies involve molecular data to reveal variation among the population as well as among species. However, molecular systematics rely largely on empirical results: therefore, increasing knowledge about rates of nucleotide change is needed to improve assumptions generally used for phylogenetic inferences and deciphering the evolutionary process within or between species. While phylogenetic relationships can be deciphered through analysis of DNA sequences among species, comparisons of DNA barcodes within species furnish information about the population structure of species and their evolutionary history.

In earthworms despite their fundamental importance in soil ecosystems, their population structure as a function of intraspecific diversity or genetic diversity is poorly understood and the amount of these studies are scanty, due to either less attention that was given to earthworms or other vertebrates were studied utmost. Presently limited investigations such as the role of glacial periods and contemporary processes like habitat fragmentation on the genetic diversity (see Table 3) of earthworms are studied based on the partial sequencing of COI gene and other markers (COII, 12S, 16S, 18S, 28S, H3, H4, tRNAs) and this has opened up new challenges in the field of population genetics. Earthworms have a complicated pattern of gene flow with a weak relationship between genetic and geographic distances.
Kautenburger (2006) studied the genetic structure of *Lumbricus terrestris* L populations at different locations in Germany and revealed an absence of isolation by distance pattern. Similar observations were inferred by Cameron et al. (2008) while investigating *Dendrobaena octaedra* populations in Alberta, Canada. They pointed out that the anthropogenic activities mainly ‘bait abandonment’ and limited active dispersal abilities lead to the significant population differentiation of *D. octaedra*. These results are related to the ideas of Sakai et al. (2001) who underlined that earthworms have limited active dispersal and it is often animal-mediated transport or limited active dispersal abilities causing genetic differentiation patterns. The genetic variations in the infieldss and the outfields of *Lumbricus rubellus*, caused by the selection of effective land-use practices (example infield eutrophication) was studied by Enckell et al. (1986) while Terhivuo & Saura (1993) stated that the high clonal diversity of *Aporrectodea rosea* is attributed to dispersal activities through agricultural practices in southern Finland. Terhivuo & Saura (1997) emphasize that human activities are the main cause of passive dispersal in *Ooctolasion cyaneum* in northern Europe. Contrary to these results the reports of Novo et al. (2009) reflected that *Hormogaster eliasae* contained cryptic species and the genetic differentiation was primarily based on the isolation by distance mechanism. The work of Torres-Leguizamon et al. (2014) on earthworm populations of *Aporrectodea icterica* reflected low genetic polymorphism and that the human-mediated favors dispersal among geographically distinct populations. Therefore these studies indicate that the population genetic structure of earthworms is strongly influenced by human activities. Giska et al. (2015) while studying the lineages of *Lumbricus rubellus* of the UK revealed that the mitochondrial lineages are deeply divergent, however not reproductive isolated and therefore may constitute a single polymorphic species rather than a complex of cryptic species. More recently, Ganin & Atopkin (2018) studied the molecular differentiation of two ecological and three color morphs of *Drawida ghilarovi*. They concluded strong genetic differentiation in two ecological forms (anecic and epigeic) with the presence of several genetic lineages in anecic forms. The genetic diversity of *Amythnas triastriatus* populations revealed two genetic lineages that were split at 2.58
Ma at the time of Quaternary glaciation in southern China as the authors (Dong et al. 2020) suggested that parthenogenesis could be an internal factor that influenced the genetic differentiation and dispersal of *A. triastriatus*. Taking together these studies, it can be anticipated that the Clitellata and in particular earthworms are heterogeneous groups and are prone to genetic differentiation. The genetic heterogeneity is due to cryptic speciation (King et al. 2008) or the amphigonic and polyploidy strains within populations (Casellato 1987). Yet, whatever the possible reasons that gave rise to genetic heterogeneity, the evolutionary and ecological consequences of its existence are ranging extensively. Furthermore, more data is required in terms of COI barcodes along with the sequencing of other mitochondrial (COI, 12S, 16S) and nuclear genes (18S, 28S) to understand how earthworms move in soils, how ecological and anthropogenic activates affect the gene flow and selection in earthworms, and how environmental stressors are manipulating the genetic differentiation in various populations of earthworm species. These studies could be essential to understand environmental changes through these ‘unsung heroes’ of the soil.

**Phylogeography and earthworm invasions**

Phylogeography is an emerging field that evaluates the geographical distribution of genealogical lineages. It is based on the analysis of DNA variations from individuals across a species range to reconstruct gene genealogies. To infer historical biogeographic events in species, phylogeography became a potent tool to understand the role of historical processes in shaping the distribution of biological species (Avise 2000). It has its role in invasion biology by improving the knowledge of invasive species. Since, the speed of invasion has dramatically increased over the past several decades due to enhanced globalization, as a result of being transported to other continents via trade either deliberately or unintentionally. This has caused the transmission of several species to other regions across water bodies where they usually are absent and now have become recognized beyond their natural ranges (Hulme 2009). Moreover, once these non-native species invade native terrestrial ecosystems, they often compete for the resources thus out-competing native species. This has attracted many ecologists and conservationists to pinpoint their concerns including alterations in native ecosystems as well threats to the native species, biodiversity, and economy (Tsutsui et al. 2000; Pejchar & Mooney 2009; Vilà et al. 2011; Qiu 2015). To overcome the invasion of these invasive species we not only need to understand their relationship with native ecosystems in terms of dynamics and establishments but also the knowledge of the history of their invasion and ecology. Nonetheless, in some instances, we even do not know the systematics of these invading species taxa (Yassin et al. 2008; Folino-Rorem et al. 2009; Bastos et al. 2011) and this makes it more problematic to predict and manage the invasion issues. Thus, the study of phylogeography is essential in the sense that it tells us the history of invasive species and the exploration of their cryptic diversity. Therefore, apart from predicting its diversity phylogeography helps in the management of the spreading of invasive species (Schult et al. 2016). Since phylogeography is based on the DNA sequences of the genome or molecular markers, the variations of patterns in DNA sequences of these molecular markers leads to the conclusions of how biogeographic events took place in all geographic scales ranging from continental to local (Avise et al. 1987; Avise 2000). Moreover, a phylogenetic tree reveals clear results of how demographic and phylogeographic forces together constitute the lineage distribution of species. Therefore, to construct a phylogeny and to depict phylogeography of taxa, the selection of accurate markers would be essential. Amongst these various molecular markers the mitochondrial genes (COI, COII, 12S, 16S) especially COI is ideal while inferring phylogeography and invasion of various terrestrial species (Chang et al. 2008; Porco et al. 2013; Shekhtovsov et al. 2018a,b). Subsequently, most of these invasions are taking place in terrestrial ecosystems therefore, it is vital to understand the ecology, population dynamics of these invading species before setting management protocols to overcome their ecological effects. Earthworms being most dominant in terrestrial soils have profound ecological consequences especially in soils where they actively participate in nutrient cycling and other soil dynamic functions (Edwards 2004). Since earthworms are an archaic invertebrate animal group, their phylogeography is quite restricted due to their little mobilities in soils and incompetency to cross rivers, seas, and mountains. However, earthworms have been widespread recently due to two main reasons: via agriculture and commerce carried by humans across the globe and secondly, the introduction of earthworms in soils for their effective functions. For example, in the coniferous forests of Finland (Huhta 1979) *Aporrectodea caliginosa* was introduced to enhance its promising results. Similarly, earthworm invasions with their middens and burrowing activities have no doubt enhance soil heterogeneity
and abundance of other soil invertebrates by creating microhabitats with larger pore sizes and high microbial biomass that attract micro and mesofauna, respectively. However, such functions are often transient, small, and restricted to soil habitats, and rather the invasion has more negative effects. For instance, the invasion of the *Amynthas* species that belong to the Asian Megascolecidae family has drawn major concerns in the United States and several studies have investigated their consequences in non-native habitats (Hendrix & Bohlen 2002; Schult et al. 2016). The study of Cameron et al. (2008) revealed single and multiple invasions of earthworm *Dendrobaena octaedra* in the boreal forest of Alberta. Similarly, Novo et al. (2015b) studied the invasion of *Amynthas* species namely *A. corticis* and *A. gracilis* in Miguel islands in the Azores. Table 4 provides details of some peer-reviewed papers on phylogeography and invasion of earthworm species. Thus, in the longer term, the invasion of non-native earthworms can have strong adverse impacts on native faunal groups. Other studies either field or laboratory-based investigations also provide strong evidence of physical disturbance to the soil, food competition, vegetation loss, alteration of organic horizons, and decline of significant micro and mesofauna in soils due to invasions (Bohlen et al. 2004a,b; Frelich et al. 2006). Thus, the concern of non-native earthworm species should be addressed primarily and more focus should be given to their population dynamics, cryptic speciation, and phylogeography to understand the network of their invasion and to overcome their consequences by providing enough unbiased sampling and DNA based datasets.

### CONCLUSION

Regardless of the fact that earthworm fauna of India is well reported as compared to other Asian Countries mainly on the basis of classical taxonomy but to solve a large number of taxonomic disagreement, an integrated approach of taxonomy may be promising in this direction. Molecular systematics of Indian earthworms is at nascent because of limited molecular database. A total of 801 DNA sequences of Indian earthworm are available on the BOLD database, while limited numbers are published yet. It is difficult to count them for correct identification unless they published. In spite of seemingly promising idea of molecular phylogenetic of earthworms a lack of comparative phylogenetic and phylogeographic inference have been observed. To overcome the current muddle of taxonomic puzzle of earthworms there is a need to move on towards integrated taxonomy.

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| Marker(s)   | Main focus                                                                 | Region(s)        | Reference                  |
|-------------|-----------------------------------------------------------------------------|------------------|---------------------------|
| COI         | Genetic structure, and invasions earthworms and Collembola                  | Europe and North America | Porco et al. 2013        |
| COI         | Introduction earthworm *Dendrobaena octaedra*                              | Northern Alberta  | Cameron et al. 2008       |
| COI/16S.    | the invasion history of *Amynthas* agrestis                                 | Northern United States | Nancy et al. 2016        |
| 16S/COI/ND1 | Systematics and phylogeography of *Metaphaire formosae* species             | Taiwan           | Chang et al. 2008         |
| COI/16S     | Historical phylogeography of *Metaphaire sieboldi*                         | Japan            | Minamiya et al. 2009      |
| COI         | Phylogeography of *E.n. nordenskioldi* populations                          | Russia           | Shekhovtsov et al. 2018a  |
| COI         | *Dendrobaena octaedra*, *Lumbricus rubellus*, and *Eisenia nordenskioldi* nordenskioldi | Eurasia          | Shekhovtsov et al. 2018b  |

Table 4. List of publications of phylogeography and invasion of earthworm using COI and other molecular markers.
Figure 1. Lay out of characterization and phylogenetic analysis of taxon using molecular markers and their applications in systematics, genetic diversity, and ecological studies of earthworms.
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