**Eisenia fetida** and **Eisenia andrei** delimitation by Automated Barcode Gap Discovery and neighbor-joining analyses: A review

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**ABSTRACT**

Identification and differentiation of morphologically similar species have been a significant challenge to taxonomists due to the higher degree of similarity in their physical appearances leading to make the taxonomic investigation more complex. Such a problem is more common in invertebrate soil animals such as earthworms (**Eisenia fetida** and **Eisenia andrei**) since their identification requires observation of morphological characters that are very difficult and complex to visualize, especially in the case of sibling or subspecies. In this review, we assessed the utility of mitochondrial cytochrome c oxidase subunit I (COI) gene as a molecular marker for identification and differentiation among these species. We achieved this by analyzing their phylogeny using the neighbor-joining method and Automated Barcode Gap Discovery (ABGD) by retrieving 84 COI sequences from NCBI. As a result, we found that the identification and differentiation success of **Eisenia fetida** was 96.42%, whereas, for **Eisenia andrei**, it was 100%. Besides, ABGD analysis suggested that the species failed to give a distinct barcode gap, and the partition pattern may be due to probable misidentification leading to generate discordance among results of ABGD and NJ tree. Finally, we suggest that the multiloci approach of the mitochondrial genome can be used to solve this taxonomic ambiguity making the molecular identification system more reliable and comprehensive fulfilling need of growing biodiversity conservation programs on a global scale.

**1. INTRODUCTION**

The soil ecosystem, which is the earth’s one of the significant ecological systems, has a crucial role in the maintenance and regulation of biogeochemical cycles. It is due to the existence of fauna and microbiota such as earthworms, microorganisms, and fungi that act as decomposers or scavengers. In addition, it is a necessary infrastructure of agricultural ecosystems on which many economically essential crop systems are dependent based on its fertility. As a result, biomonitring of the ecosystems in question is necessary to achieve the most successful farming program. In this view, it is appealed to analyze the role and efficacy of animal species with particular emphasis to earthworm species **Eisenia fetida** and **Eisenia andrei**. It is because they are biological engineers of soil comprising 90% of earth’s living mass belonging to group invertebrate [1,2]. Moreover, they are controllers of vital soil processes [3-5] which consume complex food materials present in the soil and convert them into their simple forms that can be efficiently utilized by plants. It leads to their use in vermicomposting [6]. In addition, the species under study are well-recognized soil invertebrate group in case of both ecological and toxicochemical aspects and have importance in agro-economic systems [3,5,7].

In this sequence, understanding of biodiversity and conservation of these vital biological objects is crucial to enhance the overall annual crop yields in both types of lands, that is, fertile and arid. It can be achieved by a wide range of taxon sampling of earthworms across diverse areas and their identification by available taxonomic keys. However, assigning taxa to a broad range of earthworm species (3700 described and 6000 estimated species) [8,9] distributed in terrestrial, freshwater, and marine ecosystems [10] are practically tricky. Its reason is that there is requirement to dissect genitalia of male [11,12]. Furthermore, the animals in question are very similar in their appearance, require experts that are not sufficiently available, their identification needs more labors, and are hard to identify to those who are not experts [13]. As well, underdevelopment and unavailability of morphological features in immature and damaged specimens make animal identification difficult. It makes an investigation of species diversity more challenging.

However, although the DNA based identification system in which many researchers used various molecular markers for species identification, Hebert [14] proposed the use of a partial fragment...
of mitochondrial Cytochrome c oxidase subunit I (COI), which is recently emerged and has been gaining more importance on a larger scale. Studied earthworm species are cosmopolitan [15] and traded on a commercial scale frequently and are very similar to their morphology due to which their identification is complex. Furthermore, molecular investigation of the species under study was not performed in South Africa [15] while it was delivered very less in India. Furthermore, according to Laetitia Voua Otomo et al. [15], identification as well as DNA-based distinction of Eisenia spp. would be commercially and scientifically valuable for scientists, breeders, as well as substantial buyers.

Therefore, in this review, we assessed the success rate of 84 COI gene sequences retrieved from NCBI for delimitation of these species belonging to diverse habitats and ecosystems using Automated Barcode Gap Discovery (ABGD) and neighbor-joining (NJ)-based phylogenetic analyses. It is due to the fact that these methods automatically perform computerized algorithms and have no chance of manual errors in their results. Consequently, we found that the identification and differentiation success of Eisenia fetida was 96.42%. In contrast, for Eisenia andrei, it was 100%, and there was discordance among results of ABGD and NJ tree. It may be resolved by the multiloci approach of the mitochondrial genome for species identification.

2. NOMENCLATURE

There have been complexities for naming earthworm species under study as different researchers suggested variant names to the same species. For example, following ecotoxicological investigations [16], these species were known as E. fetida or E. foetida [6] indistinctly continuing puzzlement of taxonomy [16-18]. Besides, it was not clear that which species was to be considered [6]. However, Jorge Domínguez and Manuel Aira [6] suggested that these two are phylogenetically distinct species and are reproductively isolated. Moreover, COI gene and nuclear DNA (28S)-based phylogeny validated that these species are different concerning their phylogeny [16].

3. MORPHOLOGY OF SPECIES UNDER STUDY

The morphological features of studied earthworm species are very similar [19,20] except the fact that E. fetida is banded earthworm species with the absence of pigments in the region of the groove between the segments and appear in either pale or yellow coloration. As a result, it is also called as “brandling” or “tiger” worm [21]. On the other hand, E. andrei is known as “red” earthworm which has consistent reddish coloration pattern [21] making their morphological identification system more difficult. It often leads to the generation of misleading taxonomic literature. Nevertheless, Andre [22] first stated that these species are differing morphotypes of E. fetida following their pigmentation pattern. Consequently, Bouche [23] classified these species into subspecies as E. foetida fetida and E. foetida unicolor.

Despite this fact, many researchers have considered that E. fetida and E. andrei are unique species [24]. This concept was supported by many evidences involving biochemical [25,26], spectrophotometric [27], genetically (allozyme electrophoresis) [28-30], as well as data based on reproduction [16,20,22,31]. However, genetic investigations deal with either singleton individuals or population and unable to analyze variation among individuals of the same species [21]. This generates limitations for molecular identification of these soil-dwelling animals. Because of taxonomical complications, it is needed to study these species for their differentiation [21] deeply.

4. DNA BARCODING APPROACH

Although Fragoso et al. [8] claimed that there is unavailability of easy to handle tool for species differentiation between E. fetida and E. andrei, Briones [32] proposed that such circumstances are changed. These problems of morphological identifications are complex to tackle since specialists in this area are rarely available. As a result, the mitochondrial cytochrome c oxidase subunit I (COI) gene-based identification system was developed by Hebert et al. [14] who was a remarkable milestone in the arena of biological taxonomy, which was considered as a fast and less complicated platform for species identification of earthworms [33]. It can be used for the identification of cryptic species with the potential to classify earthworms [34], assisting for the taxonomy of juveniles [25]. Furthermore, it is less severe, faster, requiring less time for data analysis and believable technique for the identification process [8]. Moreover, it is applicable for distinguishing species that are close to each other [16] and identification of earthworms from many taxa in association with morphological taxonomy [13].

Furthermore, the capacitance of the DNA barcoding tool to differentiate species has been focused on various investigations [13,35-37]. According to Lavelle et al. [7], the DNA barcode technique is a robust method for earthworm species identification that complements with the morphological classification system. Furthermore, it can be a globally necessary approach for earthworm species identification. Römbke [38] stated about assigning taxon to earthworms in association with the DNA barcoding technique along with traits that are species specific for tests related to ecotoxicology (carried out for the evaluation of environmental risks) [4,15,33]. According to Jörg Römbke et al. [38], E. fetida (found in Europe) is considered as E. andrei (distributed globally) due to their morphological similarity. However, vice versa never happens, and their identification by DNA barcoding is essential for verification of taxa during ecotoxicological tests since these species such as E. fetida (used in neurotoxicological analysis) are used as model organisms [21] in these experiments.

4.1. Species Identification

Earthworms are challenging to identify since more considerable phenotypic divergence and unavailability of easily observable external features [39] and the existence of numerous cryptic species [36,40,41]. Since earthworms of lumbricid groups are lacking in morphological characteristics that can be stable as well as less complicated for their handling, assigning taxon to them are frequently a challenging task [42]. Moreover, the taxonomical study of this group requires analysis of the clitellum concerning its position as well as configuration and related tubercular pubertal [23,43]. Furthermore, the two species under study that are considered as a model in both ecotoxicological and waste management related to organics [16,44] are well known confusing biological species in scientific literature. It leads to inappropriate identification. As a result, although identification of E. andrei was attempted by Otomo et al. and OECD Draft Document and Santocki et al. and Saitu and Nei [4,16,45,46] and E. fetida by Swiderska et al. [47], these could not be diagnosed absolutely. We found 96.42% taxonomic success for Eisenia fetida [Figure 1], whereas 100% in the case of Eisenia andrei [Figure 2]. A similar finding was reported by Römbke [38] who stated that the differentiation of E. fetida/aandrei concerning two species had generated controversies with three groups (E. andrei, E. fetida 1, and E. fetida 2) proposed by DNA barcoding.

4.2. Automated Barcode Gap Discovery (ABGD) Analysis

To study the proposed hypothesis of earthworm species E. andrei and E. fetida, we analyzed their 84 COI sequences downloaded from NCBI
analyzed the histogram distances among COI sequences of earthworm to which formation of only two clusters was expected. Additionally, we contrast to the fact that we analyzed sequences of only two species due to the analyzed sequences were belonging to four groups [Figure 4] in [Figure 3]. However, simulation of species partition showed that divergence (more elegant bulk head among species groups at prior intraspecific that initial separation was constant after the specified interval. We got where there would not be further partitioning. Furthermore, it was found that initial separation was constant after the specified interval. We got more elegant bulk head among species groups at prior intraspecific divergence (P) of 0.0046 beyond which no partitioning was possible [Figure 3]. However, simulation of species partition showed that the analyzed sequences were belonging to four groups [Figure 4] in contrast to the fact that we analyzed sequences of only two species due to which formation of only two clusters was expected. Additionally, we analyzed the histogram distances among COI sequences of earthworm species E. fetida and E. andrie [Figure 5].

4.3. Barcode Gap

We could not identify distinct barcode gap among analyzed sequences like observation reported by Wiens [48] for data of Agrodietaeus irrespective of the fact that such result was obtained when less number of specimens is analyzed per species [49]. This may be seen, in the cases, where ABGD detects hypotheses of multiple species. Shockingly, many of them may be error chrome in the instances, where COI sequences do not show complete congruence with earlier identified species boundaries usually belonging to species complexes. To add, the distribution of pairwise distances differs according to data. In some cases, an explicit barcode gap exists, and in other cases, it does not [49]. However, since simulations in ABGD work only in the presence of 3–5 sequences for each species, it is clear that the data analyzed by us passed through required simulations since it contains more than 5 sequences for each species.

4.4. Partition

We analyzed barcode sequences through their partition pattern since Puillandre et al. [49] proposed that ABGD identifies the barcode gap further than set limit of genetic divergence within species. It is utilized to make the partition of a data and then the limit of intraspecific alteration. Besides, prediction of barcode gap can be utilized recursively to the obtained clusters to get more elegant partitions till the condition arrives where there would not be further partitioning. Furthermore, it was found that initial separation was constant after the specified interval. We got more elegant bulk head among species groups at prior intraspecific divergence (P) of 0.0046 beyond which no partitioning was possible [Figure 3]. However, simulation of species partition showed that the analyzed sequences were belonging to four groups [Figure 4] in contrast to the fact that we analyzed sequences of only two species due to which formation of only two clusters was expected. Additionally, we analyzed the histogram distances among COI sequences of earthworm species E. fetida and E. andrie [Figure 5].

Two COI sequences of E. fetida (accession no., KM000898.1; KM000899.1) were clustered outside their species-specific group, whereas 12 sequences (LC006114.1, FJ214228.1, JX531566.1, EF156635.1, KX671538.1, EF077594.1, EF077593.1, KP236561.1, EF077595.1, KP236577.1, KP236563.1, and KP236562.1) of same species were grouped into cluster of E. andrie. This suggested that these sequences were either misidentified or affected by the process of divergent evolution. Moreover, the two sequences of E. fetida that showed irrelevant groupings may be either inaccurately identified or showed the highest mutation rate as compared to their counterparts, or there may be cryptic species. Nevertheless, Wiens and Servedio and De Salle [48,50] claimed that new species found by DNA barcoding is not definite and requires analysis of more factors to make delimitation of species more trustworthy. In addition, COI sequences may have many dilemmas and should be integrated with other molecular markers, external features, as well as database related with either geography or ecology to explicitly delimit related species [32,51-63]. Moreover, Valembois [64] proposed that the effectiveness of DNA barcode regions of COI genes is questioned in a few cases. Besides, ABGD partitioning of sequences, namely, LC006114.1, FJ214228.1, JX531566.1, and EF156635.1 belonging to species E. fetida showed congruence with phylogeny clade formation by NJ method [Figure 6]. It is because both of them grouped the sequences in question into a cluster of E. andrie, which was unexpected. However, we did not find congruence among these methods. Sequences KX671538.1, EF077594.1, EF077593.1, KP236561.1, EF077595.1, KP236577.1, KP236563.1, and KP236562.1 were grouped by ABGD partitioning in the cluster of E. andrie. In contrast, NJ method clustered them in the species-specific clade of E. fetida. Although ABGD grouped two COI sequences of E. fetida KM000898.1 and KM000899.1 into two distinct groups, the NJ method has grouped them into clusters of E. fetida [Figure 6]. Interestingly, according to NJ analysis, the sequences KP236577.1, KP236563.1, KP236562.1, KX671538.1, KP236561.1, EF077594.1, EF077593.1, EF077595.1, KM000898.1, KM000897.1, and KC788594.1 may be either subspecies or cryptic species of E. fetida. Following ABGD analysis, sequences of E. fetida (KP236577.1, KP236563.1, KP236562.1, KX671538.1, KP236561.1, EF077594.1, EF077593.1, EF077595.1, and EF077591.1) are grouped into a cluster of E. andrie, indicating that two studied bioinformatics tools generated different results for the same data. Finally, the sequence of E. fetida (KC788594.1) that was grouped by the NJ method in E. fetida group was grouped by ABGD into the cluster of the
same species, and this is one of the sequences that grouped into its species group in both cases. However, the authors stated that the result of partition given by ABGD analysis should not be considered as the last delimitation of species and may be used for only the first hypothesis of species partition and future research work on related aspect may be conducted. In addition, Puillandre et al. [49] claimed that less chance to find species prevails when their number is more irrespective of our analysis that included only two species with 84 sequences, and even though these could not be sufficiently differentiated by the ABGD method.

4.5. Demerits of ABGD

According to Bond and Stockman and Pérez-Losada et al. and De Queiroz [55,61,65], other data, namely, ecology and morphology are valuable for accurate species hypothesis that is required to be used with ABGD analysis. Moreover, users of this method are required to consider other supportive data to select among various generated partitions and avoiding inaccurate hypotheses, and finding believable alternative ways for the integrative approach are suggested. Although DNA-based information is a measurable baseline for the first hypothesis of species partition, genetic data sets may not be useful for information in all cases, such as in the case of considerably recently originated species. Unfortunately, considering more loci would be unable to improve the quality of results [49]. However, the authors claimed that ABGD could be a less complicated tool for splitting aligned sequence data into separate species that can be used in complementary with other supportive data because of integrative taxonomy. Nevertheless, for getting primary partition and species delimitation, ABGD requires the existence of a barcode gap [49] among analyzed sequences that were not found in our analysis. As a result, we could not get the expected partitions.

4.6. Phylogeny Analysis

Although prior studies related to allozyme genetic separation of E. fetida and E. andrie have been performed [21], taxonomists prefer phylogenetic analyses for studying species because of their operative functionality and benefits provided by statistical methods [21,66] used in them. According to Gong and Perkins and Heethoff et al. [42,67], molecular strategies generate a more appropriate description of taxa and set relationships concerning phylogeny between species of earthworms, and Pérez-Losada et al. [21] stated that molecular phylogeny analysis [54] inference of species boundaries could be achieved between many populations of earthworm species. In this context, we used neighbor-joining as a statistical method for analyzing 84 sequences of E. andrie and E. fetida for investigating the pattern of their species-specific cluster formation [Figure 6]. It is because since Saitou and Nei [68] claimed that phylogenetic tree constructed using this method could generate accurate topology. The same method was used by Huang et al. [13] for the successful identification of 86 Chinese earthworms collected from three provinces, namely, Sichuan, Hebei, and Beijing.
Figure 6: Phylogenetic analysis of 84 sequences of *E. andrei* and *E. fetida* retrieved from NCBI (Note: Sample ids in red font are misidentified)
For phylogeny analysis, we used MEGA 5 [69] with bootstrap support of 1000 replications, nucleotide as a substitution type, Kimura 2 parameter as a distance model, with transition-transversion substitution, uniform rates among sites, the homogenous pattern among lineages, and complete deletion as gap data treatment with the first, the second, and the third codon positions. Our analysis showed that three COI sequences of *E. fetida* with accession numbers LC006114.1, FJ214228.1, and JX531566.1 showed clustering with individuals of species *E. andrei* [Figure 6] although Qiu and Jänsch *et al*. and Oien and Stenersen. [20,70,71] claimed that the two species, *E. fetida* and *E. andrei*, which are reproductively isolated [24] and can be differentiated by considering their features related with morphological appearances, physiological processes, and molecular factors.

This may be as a result of the fact that *E. fetida* may be a representative of species complex [16] and the morphological differences such as yellow transverse segmental stripes present on *E. fetida* and the uniformly dark reddish appearance of *E. andrei* [38] is not enough for their differentiation since the characteristic of *E. fetida* disappears after fixation [22]. Moreover, the presence of unseen species within *E. fetida* is believable. It needs further analysis [38], whereas no cryptic species was observed in the case of *E. andrei*, although a single considerably different (>23%) haplotype in the batch of 7 was found [33]. In addition, existed taxonomical errors may be due to morphological misidentification as a result of divergent evolution, nuclear mitochondrial pseudogenes (NUMTs) contamination in sequences. Such mistakes, especially in the case of sibling species, subspecies, or cryptic species, can be resolved by the multilocus approach of the mitochondrial genome. Regardless of such controversies, Pérez-Losada *et al*. [61] concluded that *E. andrei* and *E. fetida* are distinct earthworm species concerning their phylogeny based on their DNA barcode analysis using mitochondrial COI gene and 28S DNA sequence investigations. This finding was supported by Mayr [72] who suggested that these are two distinct biological as well as phylogenetic species groups.

5. CONCLUSION

Misidentification or higher mutation rate in COI genes or the presence of siblings or subspecies prevents congruent results of ABGD and phylogeny analyses by NJ method, and there is a need to develop a more comprehensive strategy for the identification of studied earthworm species such as a multiloci approach of mitochondrial genome to avoid existing taxonomic uncertainties.

6. AUTHORS’ CONTRIBUTIONS

Rajesh Dhakane: Developed an idea and wrote the manuscript, Anant Shinde: Analyzed the data.

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8. CONFLICTS OF INTEREST

Authors declare that no conflicts of interest exist.

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