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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 a 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, biomonitoring 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 andrie*. 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 toxicological 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

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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 fetid* 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 taxa 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 taxa 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. andrie* 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*/*andrei* 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. andrie* and *E. fetida*, we analyzed their 84 COI sequences downloaded from NCBI

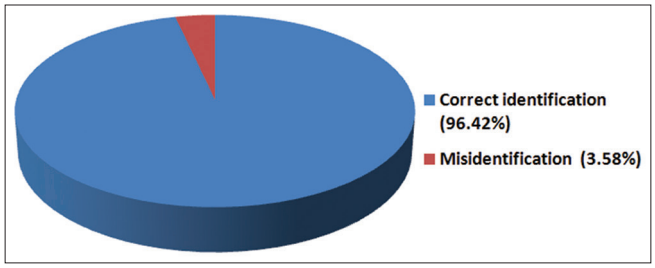

Figure 1: Species identification success (96.42%) for *Eisenia fetida*

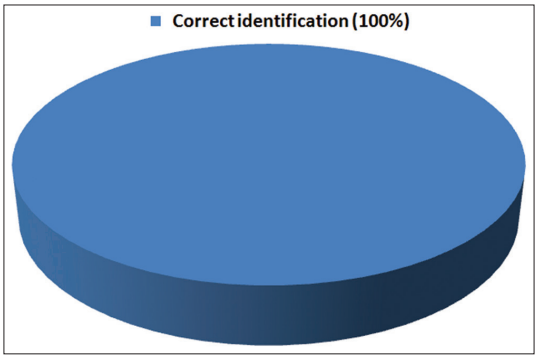

Figure 2: Species identification success (100%) for *Eisenia andrie*

through Automated Barcode Gap Discovery, which can be employed to reveal an unseen group that is used to delimit species boundaries.

4.3. Barcode Gap

We could not identify distinct barcode gap among analyzed sequences like observation reported by Wiens [48] for data of *Agrodiaetus* 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 [49], 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].

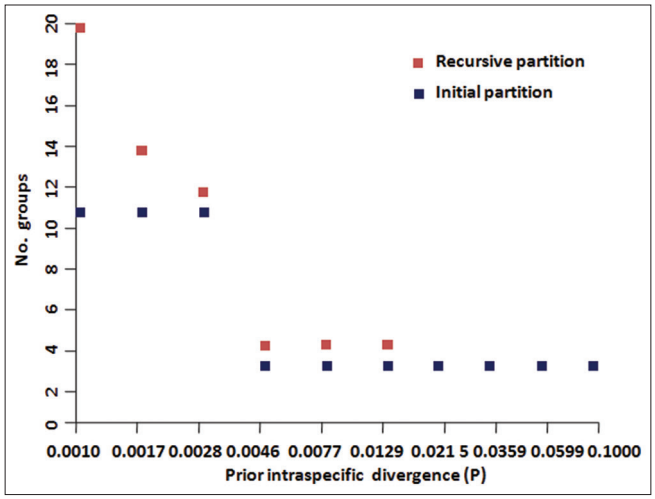

Figure 3: Initial and recursive partitions among COI gene sequences of earthworm species *E. fetida* and *E .andrie*

Two COI sequences of *E. fetida* (accession no., KM000898.1; KM0008997.1) 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. andrei*. 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 KM0008997.1 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, and EF077595.1) 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

Group[1] n: 66 ;id: KM823562.1_Eisenia_andrei LC006114.1_Eisenia_fetida FJ214228.1_Eisenia_fetida
JN870045.1_Eisenia_andrei JN870027.1_Eisenia_andrei JN870002.1_Eisenia_andrei
JX912889.1_Eisenia_andrei KM823563.1_Eisenia_andrei KX671540.1_Eisenia_andrei
KM823565.1_Eisenia_andrei KM823564.1_Eisenia_andrei JX908701.1_Eisenia_andrei
JN870062.1_Eisenia_andrei JX531566.1_Eisenia_fetida AY874508.1_Eisenia_andrei
LC006116.1_Eisenia_andrei JN870034.1_Eisenia_andrei JN870032.1_Eisenia_andrei
JN870025.1_Eisenia_andrei JX912899.1_Eisenia_andrei JX912898.1_Eisenia_andrei
JX908676.1_Eisenia_andrei JN870039.1_Eisenia_andrei JX912890.1_Eisenia_andrei
JX912888.1_Eisenia_andrei JN870031.1_Eisenia_andrei JN870015.1_Eisenia_andrei
JN870029.1_Eisenia_andrei HQ534065.1_Eisenia_andrei JX912906.1_Eisenia_andrei
KF205977.1_Eisenia_andrei EF156635.1_Eisenia_fetida KT716825.1_Eisenia_andrei
JN870063.1_Eisenia_andrei JN870059.1_Eisenia_andrei JN870049.1_Eisenia_andrei
JN870048.1_Eisenia_andrei JN870005.1_Eisenia_andrei JN870011.1_Eisenia_andrei
KX832067.1_Eisenia_andrei JN870033.1_Eisenia_andrei JN869996.1_Eisenia_andrei
JN869995.1_Eisenia_andrei KC145540.1_Eisenia_andrei KC145537.1_Eisenia_andrei
JN870003.1_Eisenia_andrei JN870001.1_Eisenia_andrei JX908645.1_Eisenia_andrei
JX908641.1_Eisenia_andrei AY874497.1_Eisenia_andrei AY874493.1_Eisenia_andrei
AY874499.1_Eisenia_andrei LC006115.1_Eisenia_andrei KC788601.1_Eisenia_andrei
KC788599.1_Eisenia_andrei DQ914627.1_Eisenia_andrei DQ914620.1_Eisenia_andrei
DQ914618.1_Eisenia_andrei KX671538.1_Eisenia_fetida EF077594.1_Eisenia_fetida
EF077593.1_Eisenia_fetida KP236561.1_Eisenia_fetida EF077595.1_Eisenia_fetida KP236577.1_Eisenia_fetida
KP236563.1_Eisenia_fetida KP236562.1_Eisenia_fetida
Group[2] n: 16 ;id: KX671544.1_Eisenia_fetida KX671543.1_Eisenia_fetida KX671541.1_Eisenia_fetida
KX671536.1_Eisenia_fetida KM823572.1_Eisenia_fetida KX671542.1_Eisenia_fetida
KM823570.1_Eisenia_fetida JX908671.1_Eisenia_fetida JX908666.1_Eisenia_fetida
EF077596.1_Eisenia_fetida AY874513.1_Eisenia_fetida KC788575.1_Eisenia_fetida KC788574.1_Eisenia_fetida
KC788584.1_Eisenia_fetida KC788589.1_Eisenia_fetida KC788594.1_Eisenia_fetida
Group[3] n: 1 ;id: KM000898.1_Eisenia_fetida
Group[4] n: 1 ;id: KM000897.1_Eisenia_fetida

Figure 4: Groups of species formed by ABGD analysis

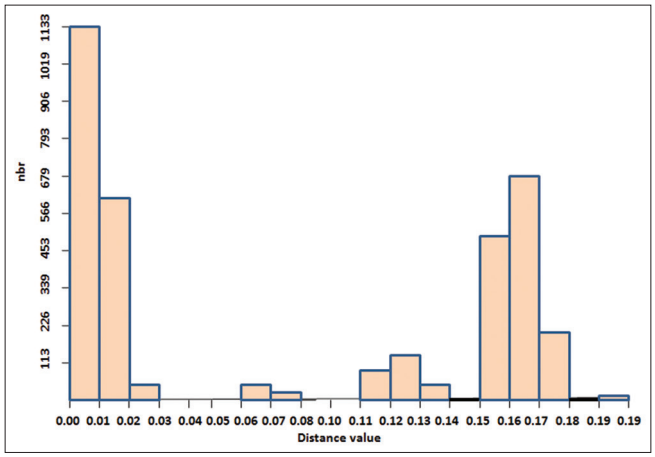

Figure 5: Histogram distances among COI sequences of earthworm species *E. fetida* and *E. andrie*

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.

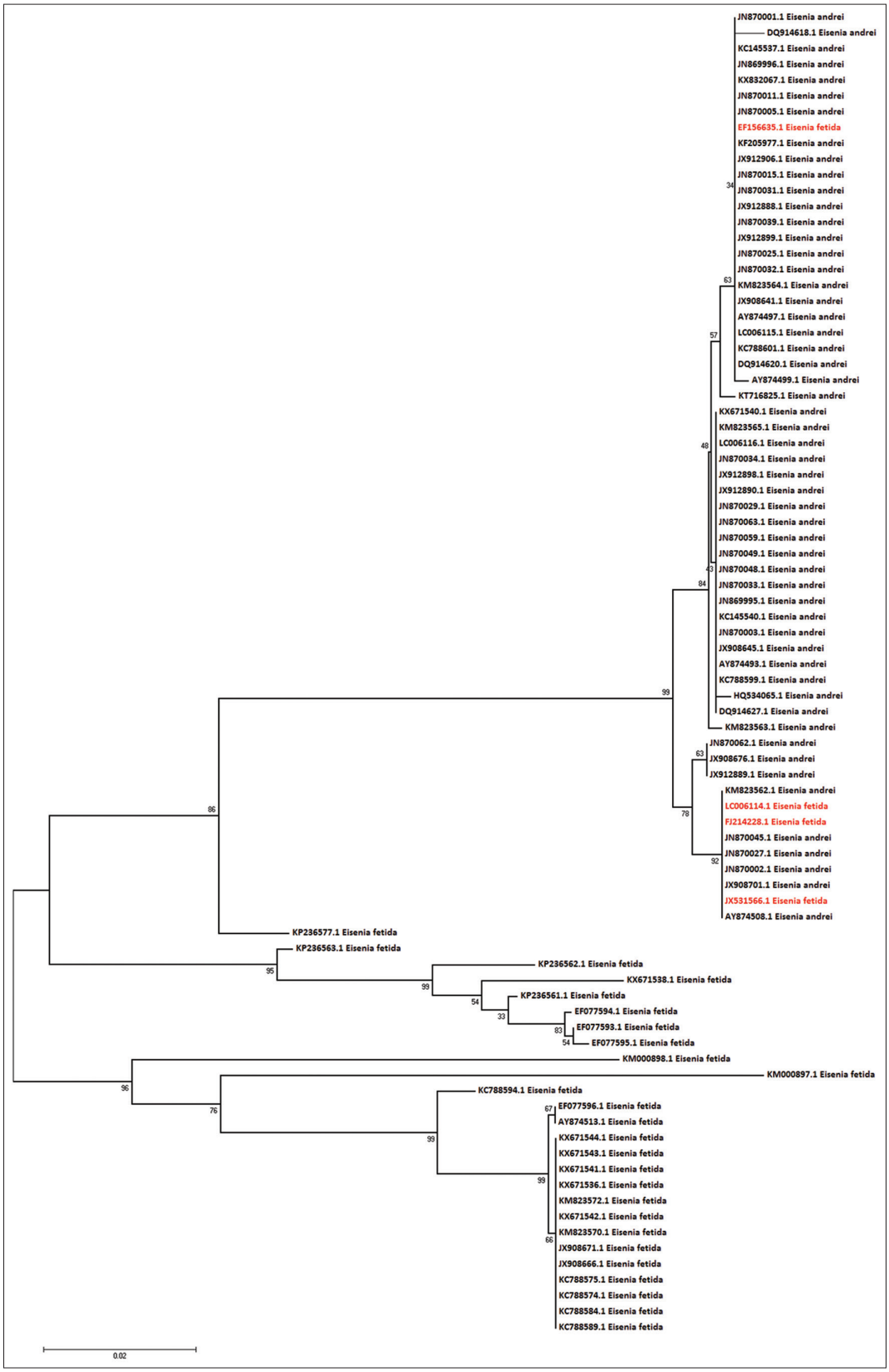

Figure 6: Phylogenetic analysis of 84 sequences of *E. andrei* and *E. fetida* retrieved from NCBI (Note: Sample ids in red font are misidentified)

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. andrie* [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.

7. ACKNOWLEDGMENT

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8. CONFLICTS OF INTEREST

Authors declare that no conflicts of interest exist.

REFERENCES

1. Decaënsa T, Porcoa D, Rougeriea R, Brown GG, James SW. Potential of DNA barcoding for earthworm research in taxonomy and ecology. Appl Soil Ecol 2013;65:35-42.
2. Edwards CA. The importance of earthworms as critical representatives of soil fauna. In: Edwards CA, editor. Earthworm Ecology. Boca Raton: CRC Press; p. 3-11.
3. Decaëns T, Jiménez JJ, Gioia C, Measey GJ, Lavelle P. The values of soil animals for conservation biology. Eur J Soil Biol 2006;42: S23-38.
4. Otomo PV, Bezuidenhout C, Maboeta MS. Inadequate taxonomy and highly divergent COI Haplotypes in laboratory and field populations of earthworms used in ecotoxicology: A case study. Afr Zool 2013b;48:290-7.
5. Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol 2011;28:2731-9.
6. Domínguez J, Aira M. Twenty years of the earthworm biotechnology research program at the University of Vigo, Spain. Int J Environ Sci Eng Res 2012;3:1-7.
7. Lavelle P, Decaëns T, Aubert M, Barot S, Blouin M, Bureau F, *et al*. Soil invertebrates and ecosystem services. Eur J Soil Biol 2006;42:S3-15.
8. Fragoso C, Freckman DW, Gupta VV, Hattori T, Hawksworth DL, Klopatek C, *et al*. Biodiversity and ecosystem function in soil. Ambio 1997;26:563-70.
9. Roch P, Valembois P, Lassegues M. Biochemical particulars of the antibacterial factor of the two subspecies *Eisenia fetida* and *Eisenia andrei*. Am Zool 1980;20:790-4.
10. Otomo PV, van Vuuren SB. Reinecke A. The usefulness of DNA barcoding in ecotoxicological investigations: Resolving taxonomic uncertainties using *Eisenia malm* 1877 as an example. Bull Environ Contam Toxicol 2009;82:261-4.
11. Tomlin AD. The earthworm bait market in North America. In: Satchell JE, editor. Earthworm Ecology. London: Chapman and Hall; 1983. p. 331-8.
12. Sims RW, Gerard BM. Earthworms. London: FSC Publications; 1999.
13. Huang J, Xu Q, Sun ZJ, Tang GL, Su ZY. Identifying earthworms through DNA barcodes. Pedobiologia 2007;51:301-9.
14. Hebert PD, Cywinska A, Ball SL, de Waard JR. Biological identifications through DNA barcodes. Proc Biol Sci 2003;270:313-21.
15. Otomo LV, Otomo PV, Bezuidenhout CC, Maboeta MS. Molecular assessment of commercial and laboratory stocks of *Eisenia* spp. (*Oligochaeta*: *Lumbricidae*) from South Africa. Afr Invertebr 2013;54:499-511.
16. OECD Draft Document; 2000. Available from: http://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?doclanguage=en&cote=env/jm/mono(2000)6.
17. Sims RW, Gerard BM. Earthworms. In: Kermack DM, Barnes RS, editors. Synopses of the British Fauna (New Series). London: Linnean Society of London and the Estuarine and Brackish-water Sciences Association; 1985.
18. Easton EG. A guide to the valid names of *Lumbricidae* (*Oligochaeta*). In: Satchell JE, editor. Earthworm Ecology from Darwin to Vermiculture. London: Chapman and Hall; 1983. p. 475-85.
19. Ross KG, Gotzek D, Ascunce MS, Shoemaker DD. Species delimitation: A case study in a problematic ant taxon. Syst Biol 2010;59:162-84.
20. Qiu JP. Earthworms and their application in environmental protection. I. Worms and their functions in the ecosystem. J Shanghai Agric Coll 1999;17:227-32.
21. Pérez-Losada M, Eiroa J, Mato S, Dominguez J. Phylogenetic species delimitation of the earthworms *Eisenia fetida* and *Eisenia andrei* (*Oligochaeta*, *Lumbricidae*) based on mitochondrial and nuclear DNA sequences. Pedobiologia 2005;49:317-24.

22. Andre F. Contribution a l'analyse experimental de la reproduction des lombriciens. Bull Biol France Belgique 1963;97:1-101.
23. Bouche MB. Lombriciens de France: Ecologie et Systematique. Paris: Institut national de la Recherche Agronomique; 1972. p. 671.
24. Domínguez J, Velando A, Ferreiro A. Are *Eisenia fetida* and *Eisenia Andrei* (*Oligochaeta*, *Lumbricidae*) different biological species? Pedobiologia 2005;49:81-7.
25. Rougerie R, Decaëns T, Deharveng L, Porco D, James SW, Chang CH, *et al*. DNA barcodes for soil animal taxonomy. Pesq Agropec Bras Brasília 2009;44:789-801.
26. Tischler W. Agraro Kologie. Jena: Gustav Fischer; 1965. p. 499.
27. Albani JR, Demuynck S, Grumiaux F, Lepretre A. Fluorescence fingerprints of *Eisenia fetida* and *Eisenia andrei*. Photochem Photobiol 2003;78:599-602.
28. Jaenike J. *Eisenia foetida* is two biological species. Megadrilogica 1982;4:6-8.
29. Henry WB. Differentiation of Allozyme Loci to distinguish between two species of *Eisenia*. M.Sc. Thesis, Mississippi State University; 1999.
30. McElroy TC, Diehl WJ. Heterosis in two closely related species of earthworm (*Eisenia fetida* and *Eisenia andrei*). Heredity 2001;87:598-608.
31. Elvira C, Domínguez J, Briones MJ. Growth and reproduction of *Eisenia andrei* and *Eisenia fetida* (*Oligochaeta*, *Lumbricidae*) in different organic residues. Pedobiologia 1996;40:377-84.
32. Briones MJ, Moran P, Posada D. Are the sexual, somatic, and genetic characters enough to solve nomenclatural problems in lumbricid taxonomy? Soil Biol Biochem 2009;41:2257-71.
33. Padial JM, Miralles A, De la Riva I, Vences M. The integrative future of taxonomy. Front Zool 2010;7:16.
34. Dickens T, Porco D, Rougerie R, Brown GG, James SW. Potential of DNA barcoding for earthworm research in taxonomy and ecology. Appl Soil Ecol 2013;65:35-42.
35. Chang CH, Rougerie R, Chen JH. Identifying earthworms through DNA barcodes: Pitfalls and promise. Pedobiologia 2009;52:171-80.
36. King RA, Tibble AL, Symondson WO. Opening a can of worms: Unprecedented sympatric cryptic diversity within British lumbricid earthworms. Mol Ecol 2008;17:4684-98.
37. Reinecke AJ, Viljoen SA. A comparison of the biology of *Eisenia fetida* and *Eisenia andrei* (*Oligochaeta*). Biol Fert Soils 1991;11:295-300.
38. Römbke J, Aira M, Backeljau T, Breugelmans K, Domínguez J, Funke E, *et al*. DNA barcoding of earthworms (*Eisenia fetida*/*andrei* complex) from 28 ecotoxicological test laboratories. Appl Soil Ecol 2015;104:3-11.
39. Shekhovtsov SV, Golovanova EV, Peltek SE. Cryptic diversity within the nordenskiold's earthworm, *Eisenia* nordenskioldi subsp. nordenskioldi (*Lumbricidae*, *Annelida*). Eur J Soil Biol 2013;58:13-8.
40. Fernandez R, Almodovar A, Novo M, Simancas B, Cosin DJ. Adding complexity to the complex: New insights into the phylogeny, diversification, and origin of parthenogenesis in the *Aporrectodea caliginosa* species complex (*Oligochaeta*, *Lumbricidae*). Mol Phylogenet Evol 2012;64:368-79.
41. James SW, Porco D, Decaens T, Richard B, Rougerie R, Erseus C. DNA barcoding reveals cryptic diversity in *Lumbricus terrestris* L., 1758 (*Clitellata*): The resurrection of L. Hercules (Savigny, 1826). PLoS One 2010;5:e15629.
42. Gong P, Perkins EJ. Earthworm toxicogenomics: A renewed genome-wide quest for novel biomarkers and mechanistic insights. Appl Soil Ecol 2016;104:12-24.
43. Sims RW. The scientific names of earthworms. In: Satchell JE, editor. Earthworm Ecology from Darwin to Vermiculture. London: Chapman and Hall; 1983. p. 467-74.
44. Chang CH, Lin YH, Chen IH, Chuang SC, Chen JH. Taxonomic re-evaluation of the Taiwanese montane earthworm *Amynthas wulinensis* Tsai, Shen and Tsai, 2001 (*Oligochaeta*: *Megascolecidae*): Polytypic species or a species complex? Org Divers Evol 2007;7:231-40.
45. Santocki M, Falniowski A, Plytycz B. Restoration of experimentally depleted coelomocytes in juvenile and adult composting earthworms *Eisenia andrei E. fetida* and *Dendrobaena veneta*. Appl Soil Ecol 2016;104:163-73.
46. Saitu N, Nei M. The number of nucleotides required to determine the branching order of three species with particular reference to the human-chimpanzee-gorilla divergence. J Mol Evol 1986;24:189-204.
47. Swiderska B, Kedracka-Krok S, Panz T, Morgan AJ, Falniowski A, Grzmil P, *et al*. Lysenin family proteins in earthworm coelomocytes-Comparative approach. Dev Comp Immunol 2016;67:404-12.
48. Wiens JJ, Servedio MR. Species delimitation in systematics: Inferring diagnostic differences between species. Proc Biol Sci 2000;267:631-6.
49. Puillandre N, Lambert A, Brouillet S, Achaz G. ABGD, automatic barcode gap discovery for primary species delimitation. Mol Ecol 2012;21:1864-77.
50. De Salle R. Species discovery versus species identification in DNA barcoding efforts: Response to rubinoff. Conserv Biol 2006;20:1545-7.
51. Wiens JJ, Penkrot TA. Delimiting species using DNA and morphological variation and discordant species limits in spiny lizards (*Sceloporus*). Syst Biol 2002;51:69-91.
52. Ahrens D, Monaghan MT, Vogler AP. DNA-based taxonomy for associating adults and larvae in multi-species assemblages of chafers (*Coleoptera*: *Scarabaeidae*). Mol Phylogenet Evol 2007;44:436-49.
53. Tsai CF, Tsai SC, Liaw GJ. Two new species of protandric pheretimoid earthworms belonging to the genus metaphire (*Megascolecidae*: *Oligochaeta*) from Taiwan. J Nat Hist 2000;34:1731-41.
54. Vences M, Thomas M, Bonett RM, Vieites DR. Deciphering amphibian diversity through DNA barcoding: Chances and challenges. Philos Trans R Soc Lond B Biol Sci 2005;360:1859-68.
55. Bond JE, Stockman, AK. An integrative method for delimiting cohesion species: Finding the population-species interface in a group of Californian trapdoor spiders with extreme genetic divergence and geographic structuring. Syst Biol 2008;57:628-46.
56. Giraud T, Refregier G, Le Gac M, de Vienne DM, Hood ME. Speciation in fungi. Fungal Genet Biol 2008;45:791-802.
57. Depraz A, Hausser J, Pfenninger M. A species delimitation approach in the *Trochulus sericeus*/*hispidus* complex reveals two cryptic species within a sharp contact zone. BMC Evol Biol 2009;9:171.
58. Damm S, Schierwater B, Hadrys H. An integrative approach to species discovery in odonates: From character-based DNA barcoding to ecology. Mol Ecol 2010;19:3881-93.
59. Goetze E. Species discovery in marine planktonic invertebrates through global molecular screening. Mol Ecol 2010;19:952-67.
60. O'Meara BC. New heuristic methods for common species delimitation and species tree inference. Syst Biol 2010;59:59-73.
61. Pérez-Losada M, Bloch R, Breinholt J, Pfenninger M, Domínguez J. Taxonomic assessment of *Lumbricidae* (*Oligochaeta*) earthworm genera using DNA barcodes. Eur J Soil Biol 2012;48:41-7.
62. Richard B, Decaens T, Rougerie R, James SW, Porco D, Hebert PD. Re-integrating earthworm juveniles into soil biodiversity studies: Species identification through DNA barcoding. Mol Ecol Resour 2010;10:606-14.
63. Will KW, Mishler BD, Wheeler QD. The perils of DNA barcoding and the need for integrative taxonomy. Syst Biol 2005;54:844-51.
64. Valembois P, Roch P, Lassegues M, Davant N. Bacteriostatic activity of a chloragogen cell secretion. Pedobiologia 1982;24:191-7.
65. De Queiroz K. Species concepts and species delimitation. Syst Biol 2007;56:879-86.
66. Vogler AP, Monaghan MT. Recent advances in DNA taxonomy. J Zool Syst Evol Res 2007;45:1-10.
67. Heethoff M, Etzold K, Scheu S. Mitochondrial COII Sequences indicate that the parthenogenetic earthworm *Octolasion tyrtaeum* (Savigny 1826) constitutes of two lineages differing in body size and

genotype. Pedobiologia 2004;48:9-13.
68. Saitou N, Nei M. The neighbor-joining method: A new method for reconstructing phylogenetic trees. Mol Biol Evol 1987;4:406-25.
69. Martinsson S, Cui Y, Martin PJ, Pinder A, Quinlan K, Wetzel MJ, *et al*. DNA-barcoding of invasive European earthworms (*Clitellata*: *Lumbricidae*) in South-Western Australia. Biol Invasions 2015;17:2527-32.
70. Jänsch S, Amorim MJ, Römbke J. Identification of the ecological requirements of important terrestrial ecotoxicological test species. Environ Rev 2005;13:51-83.
71. Oien N, Stenersen J. Esterases of earthworms-III. Electrophoresis reveals that *Eisenia fetida* is two species. Comp Biochem Physiol C Comp Pharmacol Toxicol 1984;78:277-82.
72. Mayr E. Systematics and the Origin of Species. New York: Columbia University Press; 1942.

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