DNA Barcoding: Molecular Identification and Phylogenetic Analysis of Pheretimoid Earthworm (\textit{Metaphire} sp. and \textit{Amynthas} sp.) Based on Mitochondrial Partial COI Gene from Sialkot, Pakistan

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Abstract: The extremely difficult and challenging process is identifying pheretimoid species, genus \textit{Metaphire} and \textit{Amynthas} involving increased homoplasy in various morphological characteristics. The molecular identification, phylogenetic relationships, and evolutionary divergence time of earthworms belonging to the pheretimoid complex were investigated in this study using partial mitochondrial COI (cytochrome C oxidase subunit I) gene sequences ranging from 550-680 bp. Results revealed that 86 pheretimoid earthworms were morphologically different from a total of 342 mature worms. Moreover, 11 pheretimoid species were molecularly identified, including \textit{Metaphire posthuma} (02), \textit{M. anomala} (01), \textit{M. houlleti} (02), \textit{M. californica} (01), \textit{M. birmanica} (02), \textit{Amynthas minimus} (01), \textit{A. morrisi} (01), and \textit{M. bununa} (01). A phylogenetic tree was constructed with bootstrap values of 95\%, which supported a monophyletic lineage of two well-supported clades formed by 12 partial COI sequences and 48 GenBank sequences using \textit{Hirudo medicinalis} as an outgroup. The monophyly of these obtained genera indicated overall similarity at species level. Today, species like \textit{Amynthas}, \textit{Metaphire} and \textit{Pheretima} have worm diversity in the form of pheretimoid earthworms, which dates to the Late Miocene (11.2-5.3 Mya) and the Pliocene (5.3-2.4 Mya). Compared to all relevant pheretimoid species, genetic p-distance values ranged from 0.0\% to 0.57\% (less than 1\%). These low range values demonstrated that both genera \textit{Metaphire} and \textit{Amynthas}, supported the theory, which states that there are shared similarities among the species, despite different morphology. The current study is the first attempt in Pakistan to identify earthworms through DNA barcoding thus providing a genomic stamp. The work explored the significance of COI gene sequences to construct molecular tools that will be useful to overcome the different obstacles in morphologically similar earthworm identification and their phylogenetic study.

Key words: pheretimoid earthworm, COI gene, DNA barcoding, phylogenetic analysis, molecular dating
1 Introduction
Earthworms classified as a member of the phylum Annelida (Oligochaeta, Clitellata), are the most abundant invertebrate in the soil. These are most abundant in the tropical and temperate regions, where they account for more than 80% of the biomass of the soil community[1]. There are globally 5,500 identified earthworms[2], while a total of 590 earthworm species in subcontinents, including Pakistan[3]. These soil worms colloquially referred to as soil engineers, are important for soil quality and fertility[4]. They rebalance soils by modifying their properties including physical (soil porosity, density, and texture), chemical (heavy metal reduction, cation exchange capacity, pH, soil organic matter, and nitrogen concentration), and biological (effect on microbial biota, algae growth, and fungi regeneration)[5]. Soil fields with degraded earthworm communities are identified by extremely adverse conditions such as low fertility, increased soil acidity, and decreased soil biota[6].

The pheretimoid, a model earthworm group, is native to East Asia, Southeast Asia, and Australia[7]. It is also referred vigorously as Pheretima auct or Pheretima complex[8]. In Asia, the Pheretimoid complex is one of the most diverse groups within the earthworm family Megascoliceidae, with 14 genera and over 930 species, including four new genera determined in the last decade[9,10]. Metaphire and Amynthas are two widely distributed genera in Southeast Asia, among four new Pheretimoid complex genera[10]. These two genera share similar morphological traits. In comparison, the only distinguishing feature is copulatory pouches in an area with male pores. These structures are abundant in Metaphire but not in Amynthas[11,12]. Several studies have characterized earthworms into distinct species based on their anatomical, physiological, morphological, and behavioral characteristics[13,14]. Due to the numerous physiological and morphological diagnostic keys for earthworms, proper characterization remains a significant challenge[5].

The scarcity of readily identifiable external characteristics and their considerable versatility, species distinguishing characteristics frequently rely on internal structures[6]. Additionally, the most diagnostic morphological characteristics are found exclusively in adults. As a result, the characterization of juvenile worms in various species is nearly impossible. The identification problem was addressed using the DNA-barcoding technique, categorizing adult earthworms and their juvenile forms[15,16]. Additionally, this technique can be used to identify dead earthworm specimens found in the environment and poorly preserved specimens in laboratories. DNA barcoding is a modern technique that acquires the extra benefit of being simple, accurate, and rapid in characterizing various micro and macro-organisms[17,18]. Several mitochondrial gene sequences, including NADH subunit 5, 18S rRNA, 16S rRNA, 12S rRNA, COII, and cytochrome c oxidase subunit I (COI), are used as genetic markers for species identification in a variety of invertebrates and vertebrate taxa. These sequences are approximately 530-680 bp in length[19,20], responsible for the identification of cocoon, juvenile, and adult earthworms[21,22].

Several genetic phylogenies of Megascoliceidae have been constructed and published over the last few decades[23] to resolve systematic issues among closely related species with no apparent morphological differences[24,25]. Similarly, in earthworms belonging to the pheretimoid complex, the COI gene was used to distinguish morphologically different earthworms such as Metaphire houlleti, Amynthas arenulas, and A. longiculicaulatus[26] as well as to assess the phylogenetic relationships in Metaphire and Amynthas[27]. Phylogenetic analysis indicated that a single gene or multiple genes might be useful for identifying cryptic soil invertebrate species such as earthworms[28,29]. The divergence time of Lumbricidae was previously estimated, and its chronogram indicated a late Miocene (20.5 Mya) origin[30,31]. However, the divergence time of pheretimoid species must be estimated to determine the time of diversification of Metaphire and Amynthas species within a clade.

The present study is the first attempt to determine DNA barcoding technique, phylogenetic analyses, and divergence time estimation to characterize earthworms at the molecular level. Unfortunately, despite abundant earthworm biodiversity in Pakistan, having little evidence regarding identifying earthworm species through morphological and physiological identification keys. Therefore, we aim to categorize the Asian pheretimoid earthworm complex (Metaphire sp. and Amynthas sp.) with wide genetic diversity within Zoo taxa in Pakistan on the basis of morphological and physiological aspects. Following categorization, phylogenetic trees based gene sequences were constructed to investigate inter and intraspecific relationships. In the end, molecular dating estimation for determining their origin with the time scale of the new and old clade was also performed using mitochondrial COI gene sequences.

2 Material and Methods
2.1 Specimen sampling
Earthworms were collected for identification from eight localities (Table 1) (moist and shady) along the bank sides of the Marala Ravi Link canal in the Sialkot region between May and October in 2020 (Fig. 1). Twenty soil quadruplets (four at each sampling site) were dug out with a spade with dimensions 30 × 35 × 40 cm, and unwounded, mature, clitellate earthworms were collected and sorted on the spot by hand[5,22]. The 342 sorted earthworms were placed in labelled stainless-steel containers with porous lids containing moist soil from the same locations. Earthworm containers were kept moist with water showers and were immediately transported to Microbiology Lab, Department of Zoology, G.C. University Lahore, for further investigation.
Collected species of earthworms were allowed to expel their gut material for 15 hours in a wide glass jar with a porous lid and then washed thoroughly with distilled water. These worms were preserved by immersing them in 98% alcohol for 5 minutes at room temperature. Following alcohol treatment, these worms were stored in plastic containers at 4°C morphological characterization. By observing the shape, colour, size, segmentation, clitellum, male and female genital pore, setae, prostomium, and dorsal pores at the family level (Megascolecidae), and utilizing diagrams and identification keys, the specimens were characterized and separated from each other.

2.3 DNA isolation, COI gene amplification and sequencing

Total DNA was isolated from 43 pheretimoid species for molecular identification using minor modifications. To avoid soil contamination, one cm tissue closes to the anal region of each of the morphologically identified earthworms was collected in disposable Eppendorf tubes (one for each specimen). At first, 180 μL of 1xTE buffer (1 M Tris HCl + 0.5 M ethylene-diamine-tetra acetic acid (EDTA)) + double distilled water (ddH₂O) to a volume of 1 liter was added in each Eppendorf containing earthworm tissue to solubilize and protect the DNA from degradation. Following that, 480 μL CTAB buffer (1 M Tris HCl + 5 M Sodium chloride (NaCl) + 0.5 M EDTA + 20% CTAB (cetyl-trimethyl-ammonium-bromide)) + ddH₂O to achieve a volume of 1 liter was added to the tubes. Proteinase K (25 mg/mL) was immediately added and mixed using vertex to eliminate protein-based contaminants.

To eliminate RNA contaminants, 3 μL of RNase (15 mg/mL) was added. The mixture was placed at the aqueous phase and poured into a new disposable centrifuge tube.
round of centrifugation at 14000 rpm the supernatant was discarded, and 450 μL of 80% ethanol was added. This procedure was repeated twice, and tubes were allowed to dry at a temperature of 37°C. Finally, 35 μL 1X TE buffer (containing 0.01 M tris HCl & 0.001 M EDTA) was added to dissolve the DNA and incubated for more than an hour at 37°C. Finally, 35 μL 1X TE buffer (containing 0.01 M tris HCl & 0.001 M EDTA) was added to dissolve the DNA and incubated for more than an hour at 37°C. Finally, each specimen DNA was stored at −25°C for further experiment.

DNA templates from each specimen were amplified by using the procedure with minor modifications (JCB19 (1), KPS19 (2), PVC19 (1), SKC19 (1)).

| Pheretimoid taxon (Identified) | Sampling Isolated taxon numbers (n) | Morphological features | Position of the male and female genital pore | Prostomium | Genital mark |
|--------------------------------|-------------------------------------|-----------------------|--------------------------------------------|------------|-------------|
| Metaphire posthuma (18) (Vaillant, 1868) | MCB19 (3), RJV19 (4), PVC19 (2), KPS19 (1), SKC19 (4) | 105-116 Perichaetine 12/13 Annular 14-16 4 pairs, 5/6-8/9 1 pair male pore at 18, 1 female pore at 14 Epilobic | Intra-segmental pairs at 17, 19 | | |
| Metaphire anomala (2) (Michaelsen, 1907) | JCB19 (1), SKC19 (1) | 117-123 Perichaetine 12/13 Annular 14-16 3 pairs, 5/6-7/8 1 pair male pore at 20, 1 female pore at 14 Epilobic | Found on 17-23 | | |
| Metaphire houleli (16) (Perrier, 1872) | JCB19 (2), MCB19 (2), RJV19 (1), KPS19 (1), PVC19 (1), PVC19 (5) | Dorsal side purplish brown and ventral pale, 112-118 | Male one pair at 18, 1 female pore at 14 mid ventrals Epilobic | In between spermathecal pore | |
| Amyntas minimus (11), (Horst, 1893) | MCB19 (2), JCB19 (5), PVC19 (1), KPS19 (1), SKC19 (1) | Light reddish to red, white, 80-110 Perichaetine 11/12/13 Annular 14-16 Absent 1 Male one pair at 18, 1 female pore at 14 Epilobic | Small pre-setal paired tubercles on 5-8 | | |
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2.4 Phylogenetic and divergence time analysis

COI gene sequences were obtained from pheretimoid species and compared for similarity using BLAST at NCBI site before being deposited in GenBank and assigned accession numbers. The generated and alternate homologous sequences adapted from the GenBank database were uploaded in MEGA-X 6.0.20 for alignment using the BIC (Bayesian information criterion) with the lowest score. Multiple sequence alignment was performed using the ClustalW program in MEGA-X, with a gap elongation penalty of 6.66 and a gap opening penalty of 1.5 for both multiple and pairwise alignments. Maximum likelihood and neighbour-joining methods were used to construct the phylogenetic tree, with 1000 bootstrap samples. Similarly, pairwise genetic distances of 12 pheretimoid species (2 Amynthas and 10 Metaphire) were calculated using MEGA 7.0.20.

3 Results

3.1 Morphological and molecular identification

Among 342 mature earthworm specimens collected from various locations in the Maralah Ravi Link Sialkot region, 86 morphologically distinct earthworm species were identified as pheretimoid complex, which included the highest number of Metaphire (56), the second-highest number of Amynthas (28) and the smallest number of Pheretima species (2). The Major distinct identifying features among pheretimoid species were prostomium, clitellum, positions, and number of the first dorsal, spermathecal and genital pores on the earthworm body’s outer surface. The 86 specimens of pheretimoid taxon consist of Metaphire posthuma (18), M. anomal (2), M. houlleti (16), M. californica (8), M. birmanica (12), Amynthas minima (11), A. gracilis (2), A. morrisi (11), A. diffringens (3), A. agrestis (1) and Pheretima lignicola (2). M. posthuma, and M. houlleti were observed as dominant species of earthworm complex (Table 2). Moreover, 43 COI mitochondrial gene sequences were successfully isolated from 50% of each morphologically screened earthworm species. Only 12 COI gene sequences were amplified using PCR and were thus used in subsequent studies. The failure in amplifications was attributed to DNA degradation or low-quality DNA templates due to the reaction mixture modification. All partial COI mitochondrial gene PCR amplicons were between 550-680 bp in length. PCR amplicons were sequenced and analyzed for homology using NCBI blast. These amplicons displayed a high degree of similarity to GenBank sequences for M. posthuma (02), M. anomal

Table 3: Pairwise P-genetic distances analysis for nucleotide sequences of partial COI gene of examined species of Amynthas and Metaphire genera.

| No. | Identified species | 1  | 2  | 3  | 4  | 5  | 6  | 7  | 8  | 9  | 10 | 11 | 12 |
|-----|-------------------|----|----|----|----|----|----|----|----|----|----|----|----|
| 1   | A. minimus JCB19  | 1  | 2  | 3  | 4  | 5  | 6  | 7  | 8  | 9  | 10 | 11 | 12 |
| 2   | A. morrisi JCB19  | 0.57 |    |    |    |    |    |    |    |    |    |    |    |
| 3   | M. anomal KPS19   | 0.235 | 0.38 |    |    |    |    |    |    |    |    |    |    |
| 4   | M. birmanica MCB19 | 0.205 | 0.529  | 0.206 |    |    |    |    |    |    |    |    |    |
| 5   | M. birmanica RJV19 | 0.215 | 0.527  | 0.201 | 0.084 |    |    |    |    |    |    |    |    |
| 6   | M. bununa KDS19   | 0.179 | 0.557  | 0.186 | 0.172 | 0.176 |    |    |    |    |    |    |    |
| 7   | M. californica PVC19 | 0.241 | 0.554  | 0.222 | 0.219 | 0.208 | 0.213 |    |    |    |    |    |    |
| 8   | M. houlleti PVC19  | 0.238 | 0.534  | 0.206 | 0.226 | 0.221 | 0.208 | 0.260 |    |    |    |    |    |
| 9   | M. houlleti PNV19  | 0.235 | 0.535  | 0.217 | 0.231 | 0.231 | 0.217 | 0.262 | 0.099 |    |    |    |    |
| 10  | M. posthuma KPS19  | 0.242 | 0.550  | 0.210 | 0.208 | 0.217 | 0.208 | 0.224 | 0.251 | 0.223 |    |    |    |
| 11  | M. posthuma SKC19  | 0.244 | 0.541  | 0.207 | 0.199 | 0.215 | 0.201 | 0.217 | 0.245 | 0.215 | 0.067 |    |    |
| 12  | M. sp. KPS19      | 0.190 | 0.559  | 0.219 | 0.231 | 0.217 | 0.161 | 0.249 | 0.233 | 0.247 | 0.247 | 0.238 |    |
Table 4 List of Pheretimoid earthworms with GenBank Accession Number.

| Pheretimoid species | GenBank Accession Number | Pheretimoid species | GenBank Accession Number |
|---------------------|--------------------------|---------------------|--------------------------|
| A. minimus          | *                        | A. robustus         | AB542526                 |
| A. morrisi          | *                        | P. timpoongensis    | LC127237                 |
| M. anomala          | *                        | M. schmardae        | AB425820                 |
| M. birmanica        | *                        | A. hupeiensis       | KP030717                 |
| M. birmanica        | *                        | A. benignus         | EF077563                 |
| M. bununa           | *                        | M. californica      | KC897071                 |
| M. californica      | *                        | M. tosaensis        | AB542669                 |
| M. houlleti         | *                        | A. corticis         | AB542459                 |
| M. houlleti         | *                        | A. distichus        | AB542471                 |
| M. posthuma         | *                        | M. schmardae        | EF032610                 |
| M. posthuma         | *                        | A. kiaungensis      | EF077556                 |
| Metaphire spp.      | *                        | M. californica      | KX67194                  |
| M. feijani          | AY960809                 | M. posthuma         | MT416716                 |
| M. yushii           | AY960807                 | M. posthuma         | KU565291                 |
| M. paiwanna         | AY962119                 | M. grandipenes      | KU565266                 |
| M. yuhsii           | AY739312                 | M. grandipenes      | KU565265                 |
| M. glareosa         | AY960803                 | M. birmanica        | KU565261                 |
| M. glareosa         | AY962167                 | M. birmanica        | KU565262                 |
| Megascolecidae spp. | LC319232                 | M. hijauenis        | MF479445                 |
| P. boanensis        | LC127232                 | M. houlleti         | KU565269                 |
| A. kinmenensis      | JQ936595                 | Pheretima spp.      | MF481211                 |
| M. agrestis         | AB542602                 | A. morrisi          | MH87421                  |
| M. anomala          | KU565251                 | M. birmanica        | MH845496                 |
| M. songbeensis      | MN514952                 | A. hupeiensis       | MH845523                 |
| M. yamadai          | KP030701                 | A. hupeiensis       | MH82830                  |
| A. wuhumontis       | JQ936600                 | A. hupeiensis       | MH887419                 |
| A. robustus         | KF179573                 | M. megascolidioides | AB482107                 |
| A. papulosus        | MT444906                 | A. fuscatus         | AB542478                 |
| A. morrisi          | KF205463                 | M. vesiculata       | AB542688                 |
| M. anomala          | KU565254                 | Hirudo medicinalis  | HQ33518.1                |

*means that COI sequences of these pheretimoid species have submitted to NCBI but accession numbers are still under process by GenBank. While other species that have accession numbers are adapted from GenBank.

(01), M. houlleti (02), M. californica (01), M. birmanica (02), A. minimus (01), A. morrisi (01), Metaphire spp. (01), and M. bununa (01) (Table 3).

3.2 Phylogenetic analysis

In total, 11 earthworm species were phylogenetically identified by DNA barcoding. A phylogenetic tree of the COI partial gene sequences was constructed to investigate the evolutionary relationship between 12 newly identified earthworms and 47 closely resembled pheretimoid species published in GeneBank. *Hirudo medicinalis*, a non-earthworm Annelid (HQ33518.1) (Table 4), was utilized as an out-group which was obtained from the NCBI database (Fig. 2). The Maximum likelihood phylogeny explored the monophyletic lineages for 2 well-supported clades, a simple (A) and complex (B), along with 95% bootstrap support.
and an outgroup. *A. morrisi* (KU565198.1) formed monophyletic clade A with four members of *A. hupeiensis* (NCBI database) and submerged *M. birmanica*, which was supported by 95% bootstraps. In this clade, only *M. birmanica* (*Metaphire* sp.) was merged with sister species of *A. hupeiensis* and *A. morrisi*. The result was strongly supported by 100% bootstrap. Our taxon formed a sister lineage parallel to *A. morrisi* (KU565198.1) with 87% bootstrap support. Similarly, clade B lineage, which is parallel to clade A, is further divided into sub-clades C and D with 80% bootstrap support. In this way, *M. houlleti* and *Pheretima* species shared sister lineage with 2 recently identified *M. houlleti* species in clade C (supported by 100% bootstrap). Additionally, this result confirmed that these isolated earthworm species are members of the genus *Metaphire* and *Amynthas*. While, clade D was further divided into E and F clades with 95% bootstrap level, consist of 47 taxa with three monophyletic lineages. Clade E formed a monophyletic tree with 100% bootstrap support that includes *M. posthuma* (4), *M. houlleti* (1), *M. birmanica* (3), and *M. grandipenes* (2). *M. posthuma* and *M. birmanica* had 95 and 99% similarities to GenBank species with the same name. Likewise, clade F bifurcates into two sub-clades, G and H, with 37 taxa with 90% bootstrap support, *M. bununa*, *M. anomalala*, and *A. minima* had 80% bootstrap support for their respective GenBank species (Fig. 2).

### 3.3 Evolutionary divergence time estimation

This phylogenetic clade indicated that members of Annelida phylum, specifically *H. medicinalis* (outgroup) and the Megascolecidae family (*Pheretimoid* sp.), diverged 13 Mya during the early tertiary and middle Miocene Epoch (16.4-11.2 Mya). Pheretimoid earthworms continued to diversify, giving rise to new genera like *Amynthas*, *Metaphire*, and *Pheretima* species, seven times in the late Miocene (11.2-5.3 Mya) and three times in Pliocene (5.3-2.4 Mya). As indicated by a chronogram, *M. bununa* diverged 4 Mya from a major group of *Pheretima* genus in the Early Pliocene. In comparison, *M. houlleti* was also separated from *Pheretimoid* sp. during the late Pliocene evolutionary process (2 Mya). Additionally, this result indicated that *M. bununa* was the oldest species in the chronogram, implying that it possessed more ancestral characteristics than *M. houlleti*. Moreover, *M. birmanica*, *M. californica*, *M. anomalala*, *M. posthuma*, and *M. morrisi*, and other *Pheretima* species, were diversified into their current forms during the Pleistocene Epoch the Quaternary periods (2.4-0.011 Mya). This indicates that the *Pheretimoid* sp. were regarded as modern earthworm taxa. Additionally, it was concluded from this chronogram that all evolutionary events occurred between the mid and late Cenozoic eras (Fig. 3).

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**Fig. 2** The maximum-likelihood (ML) tree for 59 pheretimoid species. Phylogenetic positions of 12 understudies (Red lineages) pheretimoid (2 *Amynthas* and 10 *Metaphire*) species with 47 adapted (Black lineages) GenBank partial COI sequence of same genera within Megascolecidae family (Table 4) (form a monophyletic group) and *H. medicinalis* (out-group) retrieved from NCBI analyzed on MEGA X software. *H. medicinalis* was used as an outgroup species. The numbers near the nodes are ML bootstrap values.
3.4 Pairwise P genetic distances
Calibrated genetic P-distances for pairwise species comparison were used to confirm the taxonomic positions of pheretimoid earthworms belonging to the *Metaphire* and *Amynthas* genera (Table 3). The pairwise distances between mitochondrial partial COI gene sequences from all concerned pheretimoid species ranged from 0.00% to 0.57% using the Tamura-Nei model on MEGA X 6.0 software and gamma distributions. The genetic p-distance values within and between species were found extremely low (less than 1%). These lowered range values confirmed the similarities within and between *Metaphire* and *Amynthas* species. Likewise, slight differences were also observed in genetic p-distance values and were corroborated by morphological characters.

4 Discussion
Total 86 earthworm species were identified on the basis of morphological and physiological characteristics in the current study. The major morpho-differentiating characteristics of pheretimoid complex earthworms were observed in their body size (75-130 cm), colour (which varies according to species), perichaetine setae, dorsal pore position, annular citellum, epibolic prostomium, spermathcal and genital pores. Various authors have also analyzed similar characteristics to aid in identifying *Amynthas* and *Metaphire* species. Members of the *Amynthas* genus were easily distinguished by their smooth, fully encircled citellum, light brightly coloured bodies. Morphologically different earthworms belong to 3 pheretimoid genera, i.e., *Metaphire*, *Amynthas*, and *Pheretima*. Some taxonomists previously reported the earthworms from Punjab province (Pakistan), including *M. posthuma*, *M. anomala*, *M. californica*, *M. houleti*, *M. gracilis*, *A. diffringens*, *A. minimus* and *P. lignicola*. While one researcher investigated three species in Gujranwala (a city of Punjab) containing *M. posthuma*, *M. birmanica*, and *P. lignicola*. Similarly, *A. morrisi*, was isolated and identified with *M. posthuma*, *A. diffringens* and *A. minimus* from different neighbouring districts of Sialkot (i.e., Narowal), Punjab. The distinguishing morphological characteristics were not sufficient shreds of evidence to categorize earthworms at the species level. The literature revealed that during interrelationship study between *Amynthas* and *Metaphire* genus, it was difficult to characterize *Amynthas* genus due to a lack of major diagnostic traits. This prompted us to use COI gene sequencing to characterize these worm species at the molecular level.

Through DNA barcoding, 11 earthworm species belonging to genera *Metaphire* and *Amynthas* were identified, while one was being unidentified. Previously researchers used COI mitochondrial gene-based DNA barcoding to molecularly characterize the *Amynthas* and *Metaphire* earthworm species in the United States, Philippine, Thailand, and China. Only eight species are classified as *Metaphire*, 3 to *Amynthas*, and one as unidentified.
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Similarly, some researchers used a 658 bp COI gene fragment at molecular level and analyze their phylogeny with divergence.

5 Conclusion

In Pakistan, this is the first study to identify earthworms at molecular level and analyze their phylogeny with divergence time. The study provides genomic stumps to 11 species of pheretimoid earthworms: *M. posthuma* (02), *M. anomalae* (01), *M. houleti* (02), *M. californica* (01), *M. birmanica* (02), *A. minimus* (01), *A. morrisi* (01) and *M. bununa* (01). The acquired data have supported the implementation of a hybrid taxonomy using classical (morphology) and modern (DNA barcoding) methods. Moreover, the phylogenetic tree has the monophyly relationship of all the species within both genera *Metaphire* and *Amythas* by utilizing COI gene sequences. The evolutionary divergence time estimation results also revealed the diversification of pheretimoid species in modern taxa such as *Amythas*, *Metaphire*, and *Pheretima* species during the late Miocene and Pliocene. However, a more detailed approach is required to examine additional samples to develop a robust taxonomy system for identifying pheretimoid earthworm species and construct a better resolved phylogenetic tree with molecular dating. It is also anticipated that mitochondrial COI sequences will help to resolve contrary taxonomy and investigate the diversity of earthworm (pheretimoid) in Pakistan.

Conflicts of Interest

The authors have reported that not a single person has a potential conflict of interest.

Competing Interests

The authors declare that they have no competing interests.

Authors Contributions

I.L. designed the study, contributed to the identification of the specimens, revised the manuscript and supervised the study. M.H. performed the genetic analyses, contributed to the collection and identification of the specimens, interpreted the data, and drafted the manuscript. M.M, K.S, A.I.D, U.Z, M.A. revised the manuscript, provided the specimen photos and performed the phylogenetic and molecular dating analysis. A.E and S.R. reviewed the taxonomic and morphological data. All authors read and approved the final version of the manuscript.

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