museu de ciències naturals de Barcelona

Animal Biodiversity and Conservation 47.1 (2024)
DOI: 10.32800/abc.2024.47.0135

Research paper

Identification of earthworms (Clitellata, Oligochaeta) from Kottayam district in Southwestern India: an integrated traditional and barcoding approach

R. Anuja[1], S. P. Narayanan[1], B. Padmakumar[1], N. V. Gopika[1], S. Kumari[2], L. Mathew[1], A. P. Thomas[1]

Author affiliations:
[1] Mahatma Gandhi University, India
[2] Career Point University, India

Corresponding author:
R. Anuja
anujar008@gmail.com

Handling Editor:
Pablo Castillo

Received: 08/02/2024
Cond. acceptance: 22/05/2024
Final acceptance: 17/072024
Published: 06/08/2024

Abstract

Identification of earthworms (Clitellata, Oligochaeta) from the Kottayam district in Southwestern India: An Integrated traditional and barcoding approach. Earthworms from various land uses in the Kottayam district of Kerala, part of the Western Ghats hotspot in India, were studied using an integrative approach that involved morphoanatomical methods and cytochrome c oxidase I (COI) barcoding techniques for the first time here. The study unveiled the presence of nine earthworm species: *Drawida ghatensis* Michaelsen, 1910; *Moniligaster julkai* Narayanan and Paliwal, 2022; *Glyphidrilus annandalei* Michaelsen, 1910; *Pontoscolex corethrurus* (Müller, 1857); *Eukerria kuekenthali* (Michaelsen, 1908); *Dichogaster bolaui* (Michaelsen, 1891); *Megascolex cochinensis* Stephenson, 1915; *M. konkanensis* Fedarb, 1898; and *Lampito mauritii* Kinberg, 1867, belonging to eight genera. The COI sequences of all nine species were deposited in GenBank. Notably, *M. julkai* and *E. kuekenthali* are novel additions to the GenBank barcode database. The Bayesian phylogram and maximum-likelihood indicated that the species *M. julkai* in this study exhibited a closer relationship to *D. ghatensis* than to other *Moniligaster* species. This study provides detailed descriptions and illustrations of all nine species, supplemented by their DNA barcodes, to facilitate future species identification.

Key words: Earthworms biodiversity, COI, DNA barcoding, Taxonomy, Western Ghats

Resumen

Identificación de lombrices (Clitellata, Oligochaeta) del distrito de Kottayam, en la India sudoccidental: Integración de métodos tradicionales y códigos de barras. Se estudiaron lombrices en tierras con distintos usos del distrito de Kottayam, en Kerala (India), una zona de western Ghats con una elevada densidad de estos animales, utilizando por primera vez una fórmula que integra métodos morfoanatómicos y técnicas basadas en el código de barras del gen de la oxidasa I del citocromo c (COI). El estudio reveló la presencia de nueve especies de lombriz: *Drawida ghatensis* Michaelsen, 1910; *Moniligaster julkai* Narayanan y Paliwal, 2022; *Glyphidrilus annandalei* Michaelsen, 1910; *Pontoscolex corethrurus* (Müller, 1857); *Eukerria kuekenthali* (Michaelsen, 1908); *Dichogaster bolaui* (Michaelsen, 1891); *Megascolex cochinensis* Stephenson, 1915; *M. konkanensis* Fedarb, 1898, y *Lampito mauritii* Kinberg, 1867, que pertenecen a ocho géneros. Las secuencias del gen COI de las nueve especies se depositaron en GenBank. Cabe destacar que *M. julkai* y *E. kuekenthali* son nuevas adiciones a la base de datos de códigos de barras de GenBank. El filograma bayesiano y el árbol de máxima probabilidad indicaron que la especie *M. julkai* en este estudio está más estrechamente relacionada con *D. ghatensis* que con otras especies de *Moniligaster*. En el estudio también se proporcionaron descripciones e ilustraciones detalladas de las nueve especies, que se complementaron con los códigos de barras del ADN, para facilitar la identificación de las especies en el futuro.

Palabras clave: Biodiversidad de lombrices, COI, Código de barras del ADN, Taxonomía, Western Ghats

Cite:
Anuja R, Narayanan SP, Padmakumar B, Gopika NV, Kumari S, Mathew L, Thomas AP, 2024. Identification of earthworms (Clitellata, Oligochaeta) from Kottayam district in Southwestern India: an integrated traditional and barcoding approach. *Animal Biodiversity and Conservation* 47.2, 135-151. DOI: 10.32800/abc.2024.47.0135

ISSN: 1578-665 X
eISSN: 2014-928 X

Introduction

Earthworms, belonging to the class Clitellata within the phylum Annelida, are widely distributed globally in most ecosystems (Philips et al 2019). To date, 5,738 species from 23 families have been described (Misirlioğlu et al 2023). Kerala is a small Indian state situated at the southern tip of the Western Ghats (between 8° 17'–12° 47' N and 74° 52'–77° 24' E), one of the world's eight 'hottest hotspots' of biodiversity (Myers et al 2000, Mittermeier et al 2011). Kerala harbors a rich array of earthworms, accounting for about 40% of the earthworm species found in the Western Ghats (Narayanan et al 2016a). The region is considered India's primary area of mega-earthworm diversity, with 271 species and subspecies identified to date (Narayanan et al 2020, 2021, 2022, 2023a, 2023b, 2024; Lone et al 2022).

Various researchers have contributed to taxonomical studies of the earthworm fauna in the state. Taxonomic studies of earthworms in Kerala began towards the end of the 20th century (Narayanan et al 2016a). According to Narayanan et al (2016a), most species studied were recorded more than 80-90 years ago, and several species in Kerala are known only from their original descriptions. Classical taxonomy relies on external morphological and internal anatomical features for to identify earthworm species. However, in some cases, it may be challenging to identify earthworms based on morphology due to the absence of stable and easily scorable diagnostic morphometric characters, especially for cryptic species that are morphologically similar but exhibit genetic variability (Reynolds 1977, Schwert 1990, Pop et al 2003, Csuzdi and Zicsi 2003). Well-known and widespread species, studied for about 100 years, have shown a high level of cryptic diversity, characterized by morphological similarities but genetic differences (King et al 2008, Rougerie et al 2009, James et al 2010, Marchán et al 2017, Shekhovtsov et al 2018). In recent times, molecular techniques have evolved into effective and precise tools to overcome the limitations of conventional identification methods, even for juveniles or cocoons (Lalthanzara et al 2018). Top of Form

DNA barcoding is a molecular taxonomic tool that relies on the use of a standardized DNA region, a short genetic marker, as a tag for rapid and accurate species identification (Hebert and Gregory 2005). Mitochondrial DNA (mtDNA) has been widely used in animal phylogenetic studies because of its faster mutation rate than nuclear DNA, leading to the accumulation of genetic differences among closely related species (Brown et al 1979, Moore 1995, Mindell et al 1997). The earthworm reference library currently comprises more than 18,000 sequences for over 1,200 species from over 49 countries, and this number is growing rapidly. Barcode data can be employed as additional support or evidence to reduce taxonomic ambiguity in the description of new species (Chang and James 2011).

Although India harbors a rich diversity of earthworms, information available regarding earthworm molecular analysis and diversity was limited until 2008 (Giraddi et al 2014). Earthworm DNA barcoding work in India is still in its initial phase, (Mathur et al 2010, Kumar et al 2013, Rao et al 2014, Thakur and Yadav 2018, Lalthanzara et al 2018, 2020, Karthigeyan et al 2019, Lone et al 2020, Vabeiryureilai et al 2020, Kumari et al 2021a, 2021b, Hijam et al 2022, Soumya et al 2022). Scientific research into classical earthworm taxonomy and genetic diversity in various unexplored soil habitat conditions of Kerala is needed as it is part of the Western Ghats biodiversity hotspot. Literature reviews have revealed only a few molecular studies on earthworms in Kerala (Kushwaha et al 2015, Thakur et al 2021, Lone et al 2022). The present study aims to provide DNA barcodes and morpho-anatomical descriptions following an integrative approach for earthworm species collected from various sites in Kottayam district, Kerala, a part of Western Ghats. It may be noted that the molecular techniques, such as DNA barcoding, potentially support traditional taxonomic studies. Additionally, such studies open the door for future interventions based on the role of cryptic species as biological tools or indicators for detecting environmental changes.

Material and methods

Study area

The study area was the Kottayam district, which is bordered by the lofty and mighty Western Ghats on the east and the Vembanad Lake and paddy fields of Kuttanad on the west. According to the documents of the Government of Kerala (GoK 2003), the district has a total area of 2,208 km^2. Kottayam district is positioned a little south of central Kerala and lies between 9.5916 °N latitude and 76.5222 ˚E longitude. The district is bound on the north by the Ernakulum district, on the east by the Idukki district, to the south by Alappuzha, and Pathanamthitta districts, and to the west by Vembanad Lake. The Kottayam district has a tropical climate with an oppressive hot season in the plains and a plentiful rainfall throughout. The district generally has an annual average rainfall of 3130.33 mm.

Collection and preservation of earthworm samples

Adult earthworms (having clitellum) were collected from different land use areas in the Kottayam district (fig. 1, table 1) using the conventional digging and hand sorting method (Andersen and Ingram 1993) with the help of a spade or shovel. The anterior part of each earthworm and the triplicates of the same species were fixed in 5% formalin for taxonomical identification or further reference. The tail regions (posterior segments) of these earthworms were preserved in 90% ethanol and kept at ambient temperature for later DNA extraction.

Taxonomic identification

All relevant morphological and anatomical characterization of the formalin preserved earthworms was carried out by dorsal dissection under a stereomicroscope (Nikon SMZ800N) with the help of monographs and following standard literature (Stephenson 1923, Gates

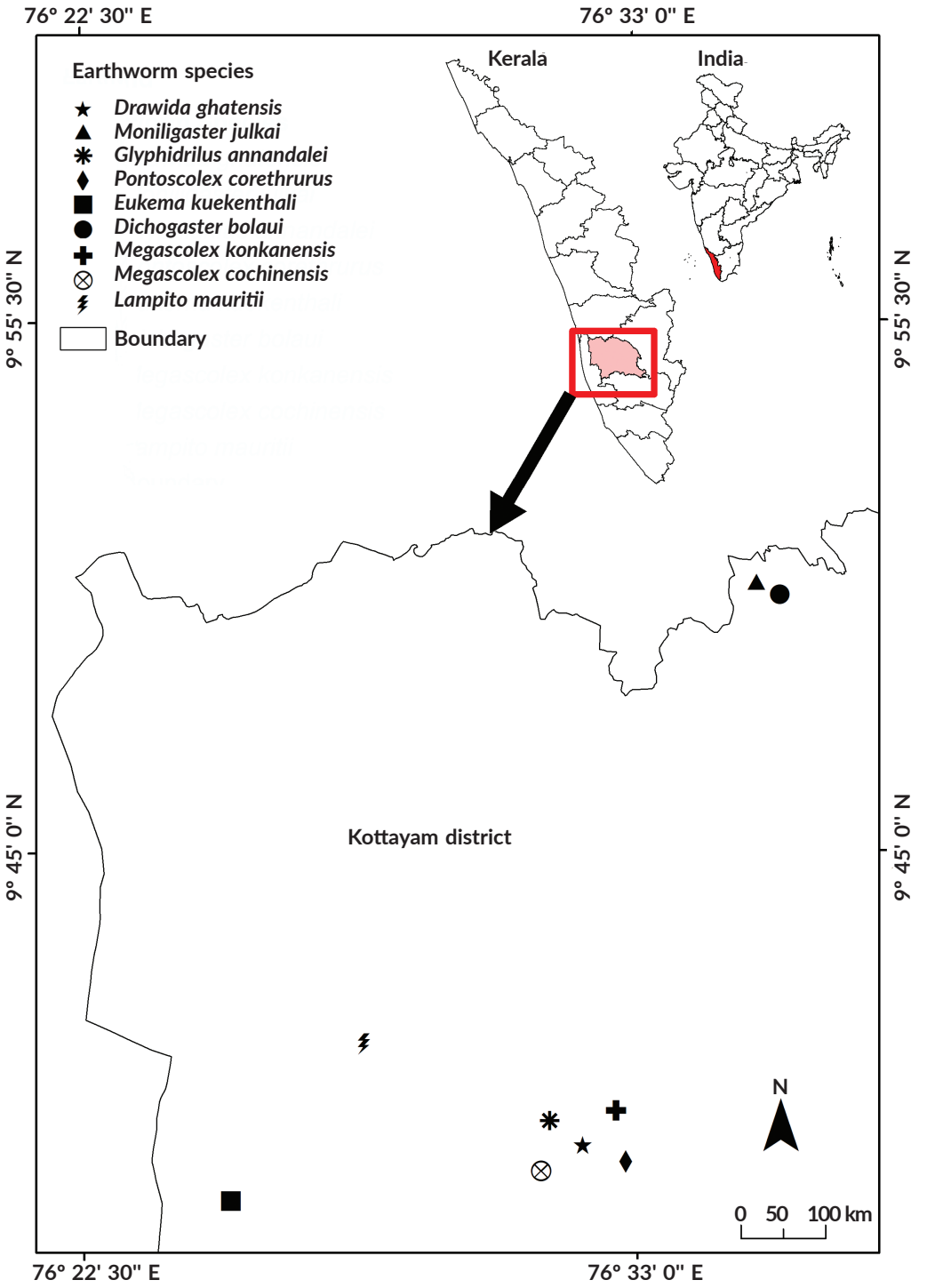

Fig. 1. Collection sites of different earthworm species from various geographical locations of the Kottayam district.

Fig. 1. Lugar de recolección de diferentes especies de lombriz de varias ubicaciones geográficas del distrito de Kottayam.

1972, Julka 1988, Blakemore 2012, Narayanan et al 2022). Illustrations were made with the help of a drawing tube attached to the microscope. Museum vouchers were prepared for each of the samples with a unique voucher number (table 1) and deposited in the earthworm laboratory and museum of the Advanced Centre of Environmental Studies and Sustainable Development, Mahatma Gandhi University, Kerala, India.

Table 1. Details of COI sequences deposited in NCBI database: GenBank, GenBank accession number.

Tabla 1. *Datos de las secuencias del COI depositadas en la base de datos del Centro Nacional de Información Biotecnológica de los Estados Unidos (NCBI): GenBank, número de acceso de GenBank.*

Family	Species	Labels	GenBank	Land use	Location
Moniligastridae	*Drawida ghatensis*	ACESSD/EW/1349	OM919723	Mixed vegetation	M. G. U. campus Athirampuzha
	Moniligaster julkai	ACESSD/EW/1362	OM100705	Rubber plantation	Puthuvely
Almidae	*Glyphidrilus annandalei*	ACESSD/EW/1350	OM100704	Paddy field	M. G. U. campus Athirampuzha
Rhinodrilidae	*Pontoscolex corethrurus*	ACESSD/EW/1407	OM100707	Mixed vegetation	M. G. U. campus Athirampuzha
Ocnerodrilidae	*Eukerria kuekenthali*	ACESSD/EW/872	OM100708	Paddy field	Cheepunkal
Benhamiidae	*Dichogaster bolaui*	ACESSD/EW/777	OM714796	Mixed vegetation	Monipally
Megascolecidae	*Megascolex cochinensis*	ACESSD/EW/1406	OM100709	Mixed vegetation	M. G. U. campus Athirampuzha
	Megascolex konkanensis	ACESSD/EW/1348	OM714798	Mixed vegetation	M. G. U. campus Athirampuzha
	Lampito mauritii	ACESSD/EW/870	OM919724	Paddy field	Perumthuruthu

DNA isolation, amplification and sequencing

The tail region of ethanol-preserved earthworm samples was used for molecular characterization. DNA was isolated following a standardized protocol using 'FFPE DNA purification kit'. PCR was carried out in an Eppendorf thermocycler (Gene Amp PCR System Model #: 9902), and Applied Biosystems (by Life technologies). The quality and approximate quantity of isolated DNA was assessed using gel electrophoresis. Sequencing reaction was done in a PCR thermal cycler (GeneAmp PCR System 9700, Applied Bio systems) using the BigDye Terminator v3.1 Cycle sequencing Kit (Applied Bio systems, USA) following the manufactures' protocol. All nine sequences of earthworms were submitted to GenBank using the SEQUIN platform to obtain accession numbers, and barcodes. The DNA barcode sequences are available on the NCBI.

Sequence alignment and data analysis

Sequence similarity searches were performed using the online tool NCBI BLAST. Based on the maximum identity score (E value) we identified top most sequences. A total of 44 mitochondrial COI sequences [nine from this study (table 1) and 35 from NCBI and free public domain of BOLD (table 2)] were aligned using Clustal W multiple sequence alignment program incorporated in BioEdit and the aligned sequences were edited manually using BioEdit 7.2 (Hall 1999). The edited sequences were converted to Nexus format using MEGA 11 (Kumar et al 2018) and a Bayesian phylogram was generated using Mr. Bayes v.3.2.2 (Ronquist et al 2012). Nucleic acid substitution model was determined by J model test (Posada 2008). Bayesian Inference analyses were carried out using the GTR+I+G model of nucleotide substitution in two independent runs; each with one heated chain and one cold chain and each run consisted of Markov Chain Monte Carlo (MCMC) methods to estimate the posterior distribution of model parameters for 2,00,000 generations. Convergence occurred when the standard deviation (SD) of split frequencies fell below 0.05; the first 25% of MCMC generations were discarded as burn-in. Posterior probability values were used to estimate branch support. Nodes with a Bayesian posterior probability (BPP) of between 0.5 and 0.74 were considered as weakly supported, 0.75 to 0.89 as moderately supported, and 0.9 to 1 as well supported (Barres et al 2013). Maximum-likelihood (ML) analyses were conducted using RAxML GUI v. 1.3 (Silvestro and Michalak 2012). An ML+ rapid bootstrap analysis was performed to find the best-scoring maximum-likelihood tree. The substitution model GTRGAMMAI was applied, and 1000 bootstrap replicates were made . The branch lengths were saved using the BS brL option. Both Bayesian and RaxML trees were visualized using Fig tree program version v.1.4.2. (Rambaut 2014).

Results

Systematic accounts

The earthworm species collected and identified from the study area are arranged family-wise. Each entry gives the information in sequence:' scientific name, type locality, type, sample ID with NCBI accession no(s); collection site, description of species and remarks. Key to the earthworm species described in the present study is also given.

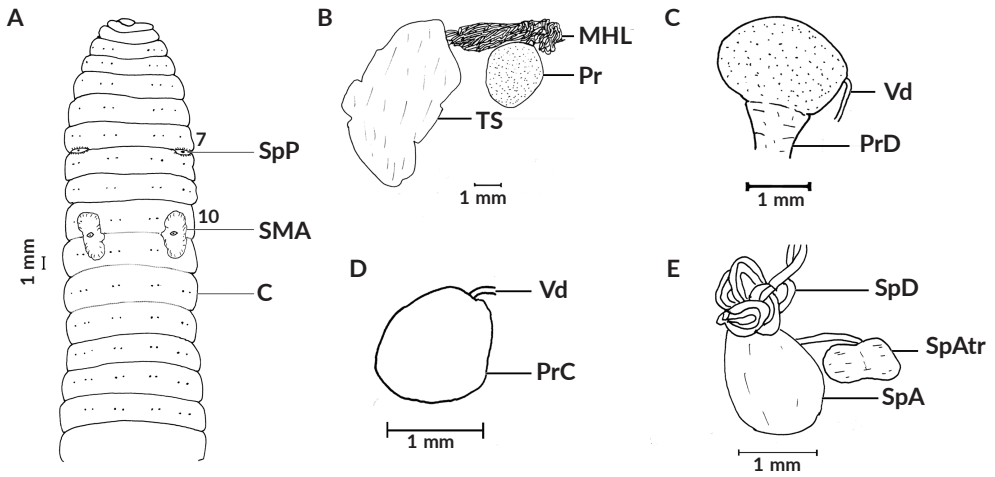

Fig. 2. *Drawida ghatensis* Michaelsen, 1910: A, ventral view; B, prostate (LHS), dorsal view; C, prostate (LHS), lateral view; D, prostatic capsule (RHS), dorsal view; E, spermathecal atrial glands (RHS), dorsal view. Abbreviations: SpP, spermathecal pores; SMA, secondary male aperture; C, clitellum; MHL, mass of Hairpin loops; Pr, prostate; TS, testis sac; Vd, vas deferens; PrD, prostatic duct; PrC, prostatic capsule; SpD, spermathecal duct; SpAtr, spermathecal atrium; SpA, spermathecal ampulla.

Fig. 2. Drawida ghatensis *Michaelsen, 1910: A, Vista ventral; B, Próstata (LHS), vista dorsal; C, Próstata (LHS), vista lateral; D, Cápsula prostática (RHS), vista dorsal; E, Glándulas del atrio de la espermateca (RHS), vista dorsal. (Para las abreviaturas, véase arriba).*

Family Moniligastridae

Drawida ghatensis Michaelsen, 1910

Type locality: Tenmalai (Thenmala), Kerala state, India (Narayanan et al 2023a).

Type: ZMUH 3596, ZSIC 4166/7 (Narayanan et al 2016a).

Sample IDs: ACESSD/EW/1349 (NCBI accession number: OM919723).

Collection site: Mahatma Gandhi University Campus (N 9° 39' 21.2" E 76° 32' 3.8"), Athirampuzha, Kottayam, Kerala, India.

Date of collection: 04/10/2021

Diagnostic features: length 60-180 mm, diameter 5-7 mm, segments 75-196. Setae lumbricine, prostomium prolobic, dorsal pore absent, clitellum annular in segments 10-13. Spermathecal pore paired, large transverse slits in intersegmental furrow 7/8, in *c* setal line. Secondary male aperture paired in intersegmental furrow 10/11, tumescences present, slightly anterior and posterior to the male pores. Genital markings absent. Gizzards 5, in segments 14-18. Testis sac paired. Vas deferens coiled in a mass of hairpin loops. Prostates paired, mushroom-shaped (lateral view), glandular, shortly stalked, erect and prostatic capsule ovoid. Spermathecae paired in segment 8, ampulla pear-shaped, duct pierces through the septum 7/8 to enter on the dorsal surface at about the center of the atrium in segment 7, atrium oval, sessile slightly projecting into segment 8 (fig. 2)

Remarks: endemic (Narayanan et al 2023a).

Moniligaster julkai Narayanan & Paliwal, 2022

Type locality: Puthuvely, Kottayam, Kerala state, India (Narayanan et al 2022).

Type: ZSI/WGRC/IR.INV.19324 (Holotype), ZSI/WGRC/IR.INV.19326 (Paratypes) (Narayanan et al 2022).

Sample IDs: ACESSD/EW/1362 (NCBI accession number: OM100705).

Collection site: Puthuvely (N 9° 50' 4.0" E 76° 35' 19.3"), Kottayam, Kerala, India.

Date of collection: 09/09/2017

Diagnostic features: length 156-238 mm, diameter 6.5-8.5 mm, segments 237-342, color bluish. Setae lumbricine, small, closely paired, clitellum annular, on segments 10-½14 (4½). Spermathecal pores paired, small transverse slits at inter-segmental furrow 7/8, aligned at *cd* setal lines. Secondary male aperture paired, in transverse slits, lateral to *b* setal lines, at intersegmental furrow 10/11. Genital markings absent. Gizzards, large, 2-3 in segments 12–16. Vas deferens a mass of hairpin loops, a mass larger than the testis sac, entering the prostate directly, a little above the ectal end in the glandular portion. Prostates glandular, tubular, slender entally, bulbous at base, duct sinuous and bulged at the base, prostatic capsule slender, tubular with smooth margins. Spermathecal atrial glands paired in segment 7, duct of each gland about five times the length of the common atrial duct, which is hidden in the parietes in segment 7. Nephridia avesiculate, functional at segment 10. Intestinal origin in segment 27 (fig. 3).

Remarks: endemic (Narayanan et al 2023a).

Family Almidae

Glyphidrilus annandalei Michaelsen, 1910

Type locality: Quilon (Kollam), Kerala state, India (Chanabun et al 2013).

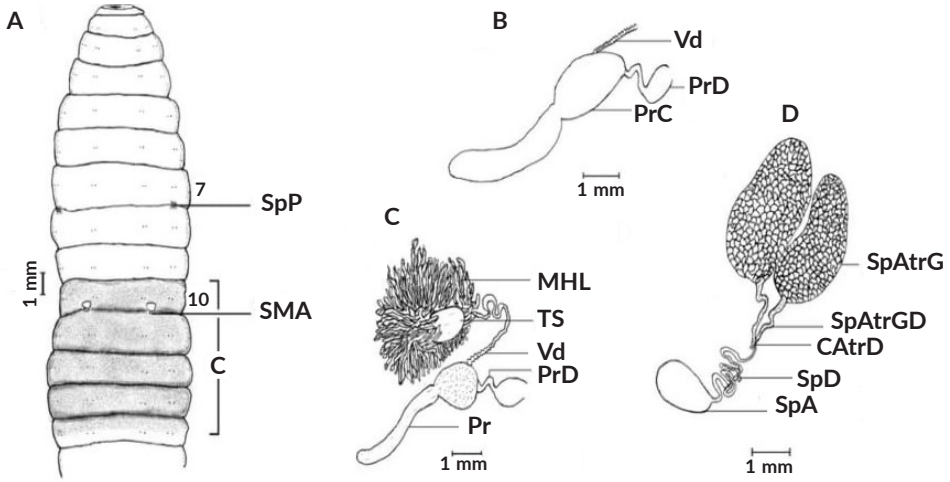

Fig. 3. *Moniligaster julkai* Narayanan & Paliwal, 2022: A, ventral view; B, prostate (RHS), dorsal view; C, prostatic capsule (RHS), dorsal view; D, spermathecae (RHS), dorsal view. Abbreviations: SpP, spermathecal pores; SMA, secondary male aperture; C, clitellum; Vd, Vas deferens; PrD, prostatic duct; PrC, prostatic capsule; MHL, mass of Hairpin loops; TS, Testis sac; Pr, prostate; SpAtrG, spermathecal atrial gland; SpAtrGD, spermathecal atrial gland duct; CAtrD, common atrial duct; SpD, spermathecal duct; SpA, spermathecal ampulla.

Fig. 3. Moniligaster julkai *Narayanan y Paliwal, 2022: A, vista ventral; B, próstata (RHS), vista dorsal; C, cápsula prostática (RHS), vista dorsal; D, espermatecas (RHS), vista dorsal. (Para las abreviaturas, véase arriba).*

Type: ZMUH 3600 (Lectotype), ZMUH 3600.1 (Paralectotype) [as ZMH] (Chanabun et al 2013).

Sample IDs: ACESSD/EW/1350 (NCBI accession number: OM100704).

Collection site: Perumthuruthu (N 9° 39' 21.2" E 76° 32' 3.8"), Kottayam, Kerala, India.

Date of collection: 04/10/2021

Diagnostic features: length 125 mm, diameter 3 mm, segments 220. Setae lumbricine, widely paired as far as 12, prostomium zygolobous, dorsal pores absent, clitellum ring-shaped beginning in 17/18-36. Wings run from segments 25, 27, 28-32, 33, wing ridges are bent downwards somewhat towards the body wall. Male pores are two point-like depressions in 29/30. Spermathecal pores in groups of one to six, with the arrangement characteristic of the genus, intersegmental septa 6/7-11/12 thickened. Genital markings laterally paired or asymmetrical on *bc* setal line in segments 18-26 and 33, median, unpaired on *aa* setal line in segments 13-17. Gizzard fairly large, in segment 8, anterior end apparently getting into segment 7. Last pair of heart in segment 11. Prostates apparently absent. Ovisacs may be present in segment 14. Spermathecae simple, thickly pear-shaped or spherical, with short and narrow stalk, sessile in appearance, the duct being embedded in the body wall. Intestinal origin in segment 25 (fig. 4).

Remarks: endemic (Narayanan et al 2023a).

Family Rhinodrilidae

Pontoscolex corethrurus (Müller, 1857)

Type locality: grass lawn behind the Museu de Ecologia Fritz Müller (former house of Fritz Müller), Blumenau, Santa Catarina, Brazil (Neotype) (James et al 2019).

Type: MZUSP 3532 (Neotype) (James et al 2019).

Sample IDs: ACESSD/EW/1407 (NCBI accession number: OM100707).

Collection site: Mahatma Gandhi University campus (N 9° 39' 21.2" E 76° 32' 3.8"), Athirampuzha, Kottayam, Kerala, India.

Date of collection: 04/10/2021

Diagnostic features: length 34-86 mm, diameter 3 mm, segments 121-190. Setae lumbricine at the anterior portion and gradually irregular and becoming quincunx towards the posterior end. Prostomium pointed, usually retracted on preservation. Dorsal pores absent, clitellum saddle shape, in segments 14, 15-22. Segment 9, 10 contain a thin pair of lateral vessel. Strong pairs of hearts in seg. 11 and 12. Tubercula pubertatis longitudinal bands in segments 18-21. Gizzard single, spherical and muscular in segment 6. Prostates absent. Genital markings in segment 20 and 21. Male pores are not clearly defined. Female pores are minute, unorganized. Spermathecal pores are minute in inter segmental furrow 6/7/8/9. Three pairs of spermathecae in segment 7-9 each flattened disc or flask entally on a long duct adiverticulate. Intestinal origin in segment 16 (fig. 5).

Remarks: exotic (Blakemore 2012).

Family Ocnerodrilidae

Eukerria kuekenthali Michaelsen, 1908

Type locality: St. Thomas Island, West Indies.

Type: Berlin and Hamburg Museums transferred to Wrocla: 515 (Blakemore 2012).

Sample IDs: ACESSD/EW/872 (NCBI accession number: OM100708).

Collection site: Cheepunkal (N 9° 38' 26.2" E 76° 25' 16.9"), Kottayam, Kerala, India.

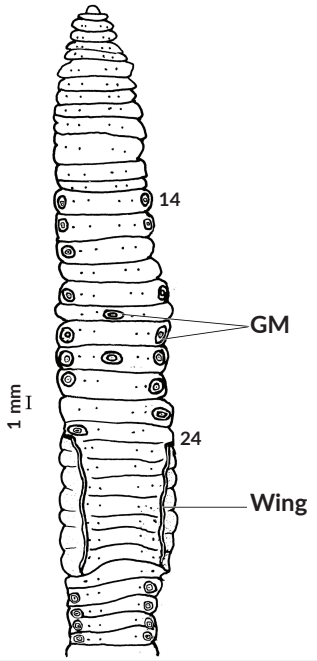

Fig. 4. *Glyphidrilus annandalei* Michaelsen, 1910, ventral view. Abbreviations: GM, genital marking.

Fig. 4. Glyphidrilus annandalei *Michaelsen, 1910, vista ventral: G.M., Marcas genitales. (Para las abreviaturas, véase arriba).*

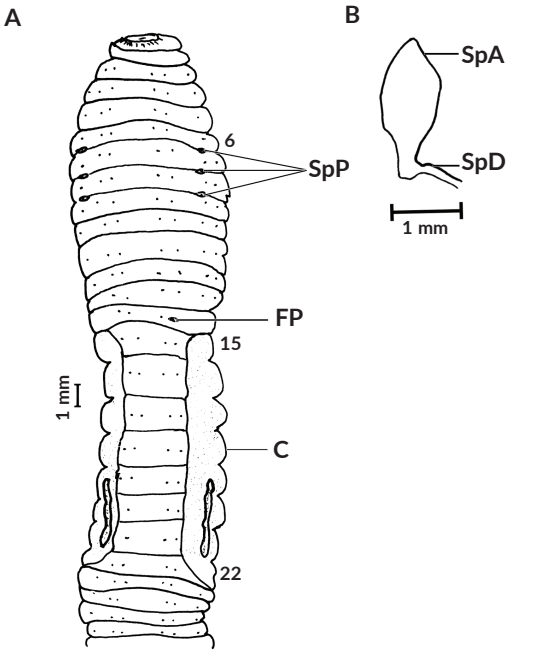

Fig. 5. *Pontoscolex corethrurus* (Müller, 1857): A, ventral view; B spermathecae (LHS). Abbreviations: SpP, spermathecal pores; FP, female pore; C, clitellum; SpA, spermathecal ampulla; SpD, spermathecal duct.

Fig. 5. Pontoscolex corethrurus ***(Müller, 1857): A, vista ventral; B, espermatecas (LHS).*** *(Para las abreviaturas, véase arriba).*

Date of collection: 11/12/2017

Diagnostic features: length 35-40 mm, diameter 1 mm, segments 82-93, red. Setae closely paired (lumbricine arrangement), clitellum is annular but thinner and colorless extends in 13,14-19,20th segment, prostomium prolobic. Dorsal pores are absent. Muscular septa are present in all intersegmental furrows. Hearts 3, in segment 9-11. A Small gizzard is in the 7th segment. Male pores, are paired at segment 18 and just lateral to the *b* setal line. Female pores are minute, paired at segment 14 in the *b* setal line. Spermathecal pores in intersegmental furrow 7/8/9 in *ab* setal line. Genital marking in segment 21, with a pore on each side just lateral to the *b* setal line. Prostates, long, duct 1½-1 mm long. Spermathecae adiverticulate in segments 8 and 9, duct as long as ampulla (fig. 6).

Remarks: exotic (Blakemore 2012).

Family Benhamiidae

Dichogaster bolaui (Michaelsen, 1891)

Type locality: Bergedorf (53.483 °N, 10.216 °E), Germany.

Type: ZMUH 285, BMNH 1924:3:1:244, MNHU 7334, MZUT 52, NHRS 1247, RNHL, USNM 34166 (Reynolds and Wetzel 2019).

Sample IDs: ACESSD/EW/777(NCBI accession number: OM714796).

Collection site: Monipally (N 9° 49' 49.9" E 76° 35' 45.8"), Kottayam, Kerala, India.

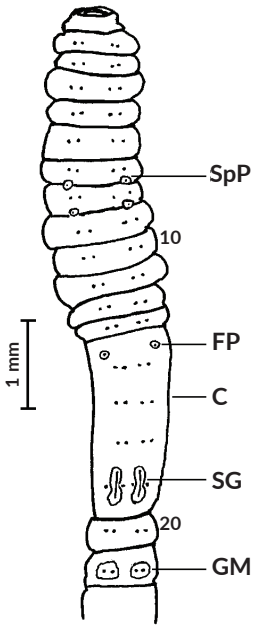

Fig. 6. *Eukerria kuekenthali* (Michaelsen, 1908), ventral view. Abbreviations: SpP, spermathecal pores; GM, genital marking; FP, female pore; C, clitellum; SG, seminal groove.

Fig. 6. Eukerria kuekenthali *(Michaelsen, 1908), vista ventral. (Para las abreviaturas, véase arriba).*

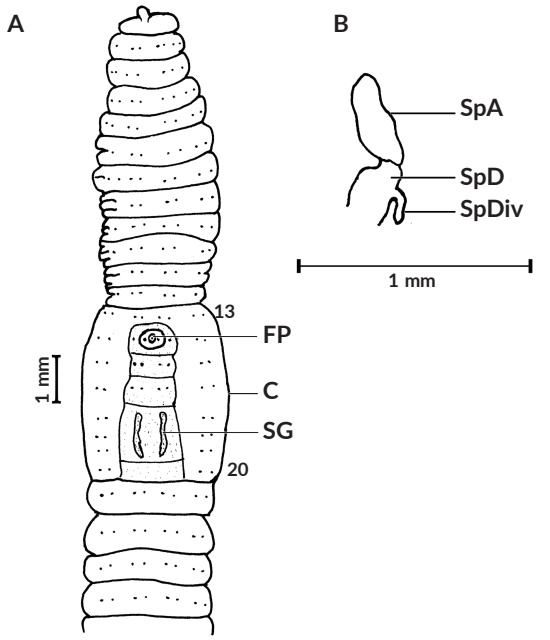

Fig. 7. *Dichogaster bolaui* (Michaelsen, 1891): A, ventral view; B, spermathecae (LHS). Abbreviations: FP, female pore; C, clitellum; SG, seminal groove; SpA, spermathecal ampulla; SpD, spermathecal duct; SpDiv, spermathecal diverticulum.

Fig. 7. Dichogaster bolaui *(Michaelsen, 1891): A, Vista ventral; B, Espermatecas (LHS). (Para las abreviaturas, véase arriba).*

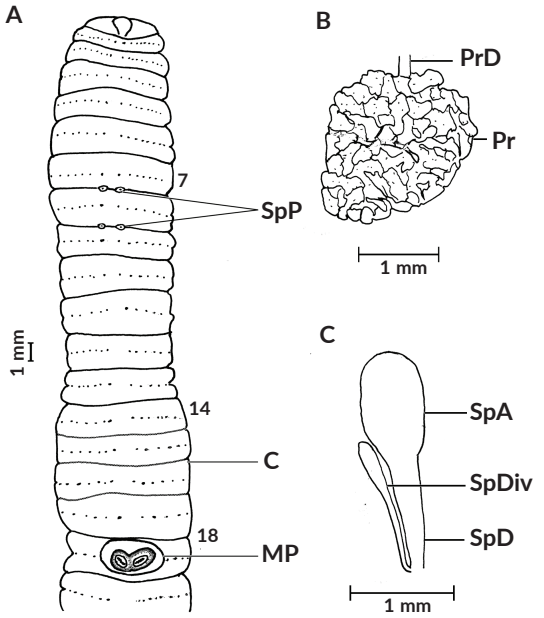

Fig. 8. *Megascolex cochinensis* Stephenson, 1915: A, ventral view; B, prostate (RHS); C, spermathecae (RHS). Abbreviations: SpP, spermathecal pores; C, clitellum; MP, male pore; PrD, prostatic duct; Pr, prostate; SpA, spermathecal ampulla; SpDiv, spermathecal diverticulum; SpD, spermathecal duct.

Fig. 8. Megascolex cochinensis *Stephenson, 1915: A, Vista ventral; B, Próstata (RHS): C, Espermatecas (RHS). (Para las abreviaturas, véase arriba).*

Date of collection: 10/05/2017

Diagnostic features: length 18-32 mm, diameter 1 mm, segments 53-92. Setae lumbricine, prostomium epilobous, dorsal pore starts at inter-segmental furrow 5/6 or 6/7, clitellum saddle-shaped, thinner at ventral portion. Male pores minute, in seminal grooves, on the setal arc of segment 18. Seminal grooves slightly concave between setal arcs of segments 17 and 19. Genital marking absent, two forms of penial setae are present. Spermathecal pores two pairs at intersegmental furrows 7/8/9 at or near setal line *a*. Female pore single, median, presetal. Gizzards in segments 6, 7. Spermathecal duct barrel-shaped, about the same size of the ampulla. Intestinal origin in segments 17 or 19 (fig. 7).

Remarks: exotic (Blakemore 2012).

Family Megascolecidae

Megascolex cochinensis Stephenson, 1915

Type locality: Forest Tramway (10.301°N, 76.592°E), Kerala state, India.

Type: ZSIC 6593 (Reynolds and Wetzel 2019).

Sample IDs: ACESSD/EW/406 (NCBI accession number: OM100709).

Collection site: Mahatma Gandhi University campus (N 9° 39' 21.2" E 76° 32' 3.8"), Athirampuzha, Kottayam, Kerala, India.

Date of collection: 04/10/2021

Diagnostic features: length 46-165 mm, diameter 2 mm, segments 56-166. Setae perechaetine, setae closer, set ventrally, prostomium epilobous, clitellum annular, from segments 14 to 2/3 17. First dorsal pore from intersegmental furrow 5/6. Male pores on sement 18 as oblique wavy slits, the posterior ends of which approach each other on a white oval elevation, also oblique , almost touching in the middle line, the area surrounding the papillae depressed, and the whole surrounded by an oval wall, penial setae absent. Female pores single, on segment 14. Spermathecal pores two pairs, in intersegmental furrows 7/8/9, in line with *a* setal lines. Gizzard large, barrel-shaped, in segment 5. Last pair of heart in segment 13. Seminal vesicles, racemose, moderately large, in segments 11 and 12. Prostates in segment 18, each a mass of small rounded lobules, duct passing straight inwards. Spermathecal ampulla ovoid, duct as long as ampulla and less than half as wide, diverticulum arising from ectal end of duct, club-shaped, reaching about middle of ampulla. Intestinal origin in segment 19 (fig.8).

Remarks: endemic (Narayanan et al 2023a).

Megascolex konkanensis Fedarb, 1898

Type locality: somewhere in North Konkan, Maharashtra state, India.

Type: ? (Reynolds and Wetzel 2019).

Sample IDs: ACESSD/EW/1348 (NCBI accession number: OM714798).

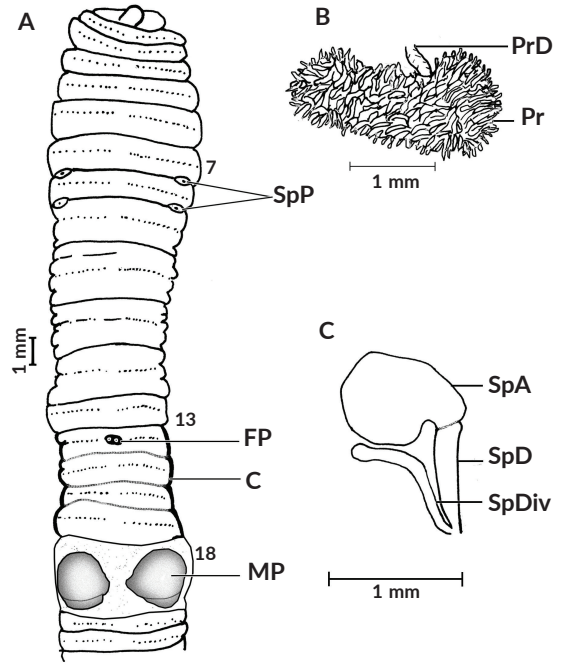

Fig. 9. *Megascolex konkanensis* Fedarb, 1898: A, ventral view; B, prostate (RHS); C, spermathecae (RHS). Abbreviations: SpP, spermathecal pores; FP, female pores; C, clitellum; MP, male pore; PrD, prostatic duct; Pr, prostate; SpA, spermathecal ampulla; SpD, spermathecal duct; SpDiv, spermathecal diverticulum.

Fig. 9. Megascolex konkanensis *Fedarb, 1898: A, Vista ventral; B, Próstata (RHS); C, Espermatecas (RHS). (Para las abreviaturas, véase arriba).*

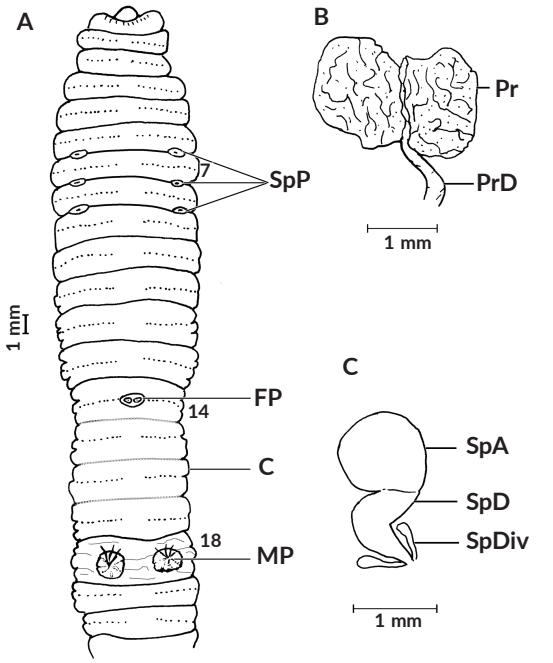

Fig. 10. *Lampito mauritii* Kinberg, 1867: A, ventral view; B, prostate (LHS); C, spermathecae (RHS). Abbreviations: SpP, spermathecal pores; FP, female pore; C, clitellum; MP, male pore; Pr, prostate; PrD, prostatic duct; SpA, spermathecal ampulla; SpD, spermathecal duct; SpDiv, spermathecal diverticulum.

Fig. 10. Lampito mauritii *Kinberg, 1867: A, Vista ventral; B, Próstata (LHS); C, Espermatecas (RHS). (Para las abreviaturas, véase arriba).*

Collection site: Mahatma Gandhi University campus (N 9° 39' 21.2" E 76° 32' 3.8"), Athirampuzha, Kottayam, Kerala, India.

Date of collection: 04/10/2021

Diagnostic features: length 78-250 mm, diameter 2 mm, segments 216-234. Long worm, anterior end truncate. Setae perechaetine, prostomium epilobous, clitellum annular, in segments 14–16. Dorsal pores start in intersegmental furrow 4/5. Male pores each in a special area, which when fully developed is oval in segment 18, penial setae absent. Female pores, paired, on segment 14. Spermathecal pores two pairs, in intersegmental furrows 7/8/9. Gizzard large, in segment 6. Intestine begins in segment 16. Last pair of heart in segment 13. Prostates mop-like, duct thick and fairly long, muscular and thinner at the ends. Spermathecae stalked, ampulla pear-shaped, diverticulum present, arising from the ectal end, slightly swollen at ental end, about half length of the ampulla. Intestinal origin in segment 16 (fig. 9).

Remarks: endemic (Narayanan et al 2023a).

Lampito mauritii Kinberg, 1867

Type locality: Mauritius.

Type: NHRS 162 (Reynolds and Wetzel 2019).

Sample IDs: ACESSD/EW/870 (NCBI accession number: OM919724).

Collection site: Perumthuruthu (N 9° 41' 22.7" E 76° 27' 49.5"), Kottayam, Kerala, India.

Date of collection: 11/12/2017

Diagnostic features: length 66-125 mm, diameter 3-4 mm, segments 178-179. Setae perichaetine, some ventral setae on the anterior part of the body much enlarged, prostomium epilobous, clitellum annular, in segments 14-17. First dorsal pore, in intersegmental furrow 10/11 or 11/12. Gizzard in segment 5. Male pores on segment 18. Female pores, paired, on segment 14, penial setae present. Spermathecal pores three pairs, in intersegmental furrows 6/7/8/9. Prostates large racemose in segments 18-19, with muscular duct. Spermathecae with long elongated ampulla, constricted in the middle, and narrowing towards the external opening; duct not distinctly marked off, 2 club-shaped diverticula, opposite each other, one-third as long as ampulla. Meganephridia accompany the micronephridria from segment 20 onwards. Intestinal origin in segment 15 (fig. 10).

Remarks: native peregrine (Narayanan et al 2023a).

COI sequencing results

In this study, a phylogenetic analysis was conducted on nine earthworm species, representing eight genera. Among these, three species belonged to the family Megascolecidae (*M. cochinensis*, *M. konkanensis*, *L. mauritii*), two spe-

Key to the earthworm species described in the present study from Kottayam district, Kerala.

Clave para las especies de lombrices de tierra descritas en el presente estudio en el distrito de Kottayam, Kerala.

1.	Setal arrangement lumbricine	2
	Setal arrangement perichaetine	7
2.	Lumbricine regular throughout body	3
	Lumbricine at anterior portion, posterior portion quincunx arrangement	*Pontoscolex corethrurus* (Müller, 1857)
3.	Dorsal pores present	*Dichogaster bolaui* (Michaelsen, 1891)
	Dorsal pores absent	4
4.	Spermathecal atrium present	5
	Spermatheca simple, atrium absent	6
5.	Spermathecal atrium with Y-shaped atrial glands	*Moniligaster julkai* Narayanan and Paliwal, 2022
	Spermathecal atria lack Y-shaped atrial glands	*Drawida ghatensis* Michaelsen, 1910
6.	Male pores conspicuous on 18 in seminal grooves; prostatic pores in 17 and 19; clitellum on segments 13-20	*Eukerria kuekenthali* (Michaelsen, 1908)
	Male pores inconspicuous or difficult to recognize; clitellum on segments 13-18 to 35-41, laterally flared into wings in segments 25-33	*Glyphidrilus annandalei* Michaelsen, 1910
7.	Penial setae present; spermathecae bidiverticulate	*Lampito mauritii* Kinberg, 1867
	Penial setae absent; spermathecae unidiverticulate.	8
8.	Prostate racemose, mop-like; intestinal origin in segment 16	*Megascolex konkanensis* Fedarb, 1898
	Prostate racemose, not mop-like; intestinal origin in segment 19	*Megascolex cochinensis* Stephenson, 1915

cies belonged to the family Moniligastridae (*D. ghatensis*, *M. julkai*), and one species each belonged to the families Almidae (*G. annandalei*), Rhinodrilidae (*P. corethrurus*), Ocnerodrilidae (*E. kuekenthali*), and Benhamiidae (*D. bolaui*). The COI gene exhibited a unique barcode for each species. These nine COI sequences obtained were not pseudogenes as they lacked stop codons or frame shifts. No areas of uncertain alignment were identified. All sequences obtained ranged from 490 to 600 base pairs in length. The DNA sequences (COI) for all nine species were deposited in GenBank, and the accession numbers are provided in table 1. The previously mentioned DNA sequences and specimen details are included below as evidence of the information available in the public database.

The phylogenetic analysis included species from NCBI that displayed more than 90 % similarity to the species we deposited during BLAST similarity searches (fig. 11, 12). The posterior probability for each node is indicated next to the branches where the related taxa are grouped together. The evolutionary tree is drawn to scale, with branch lengths in the same units as those used to infer the phylogenetic tree. The analysis focused on a total of forty-four mitochondrial COI sequences, comprising nine sequences from species belonging to six families in the present study (table 1) and thirty-five sequences retrieved from NCBI-GenBank. *Carpetania elisae* (Hormogastridae Family) was used as the outgroup (table 2).

In both the Bayesian and ML phylograms for all sequences, two clades were observed: the first cluster included all *P. corethrurus* species from the family Rhinodrilidae, while the other cluster encompassed all other species examined in the study (fig. 11, 12). In both trees, closely related species were grouped together and distinguished from other species. Notably, *Moniligaster julkai* and *E. kuekenthali*, from this study have been added as new entries to the GenBank barcode database. The final dataset contained a total of 477 positions. Out of 477 sites, 277 are conserved, 200 are variable, including 190 parsimony-informative sites, and 10 are singleton sites.

Discussion

Among the nine earthworms collected, five species are endemic to Southwestern India (*D. ghatensis*, *M. julkai*, *G. annandalei M. cochinensis*, and *M. konkanensis*), one is a native peregrine (*L. mauritii*), and three are exotic species (*P. corethrurus*, *D. bolaui*, and *E. kuekenthali*). *Moniligaster julkai* is a recently described species from Puthuvely (Narayanan et al 2022, Anuja et al 2023). This species forms a new addition to the GenBank barcode database. *D. ghatensis* and *M. cochinensis* are mainly endemic to Kerala state, with a few records from the nearby states (Narayanan et al 2023a) and its presence in the Kottayam district of the Kerala state was already reported by earlier workers (Michaelsen 1910, Narayanan et al 2014). *G. annandalei* was recorded for the first time from Kottayam district indicating its wide distribution in the region (Anuja et al 2023). This species was found confined to wetlands, especially paddy fields. Previously, it was reported from Kozhikode, Malappuram, Pathanamthitta, Kollam, and Thiruvananthapuram Districts (Narayanan et al 2014, Deepthi and Kathireswari 2016, Sathrumithra et al 2018). *M. konkanensis* is widespread throughout Kerala state and Southwestern India (Narayanan et al 2016a, 2023a).

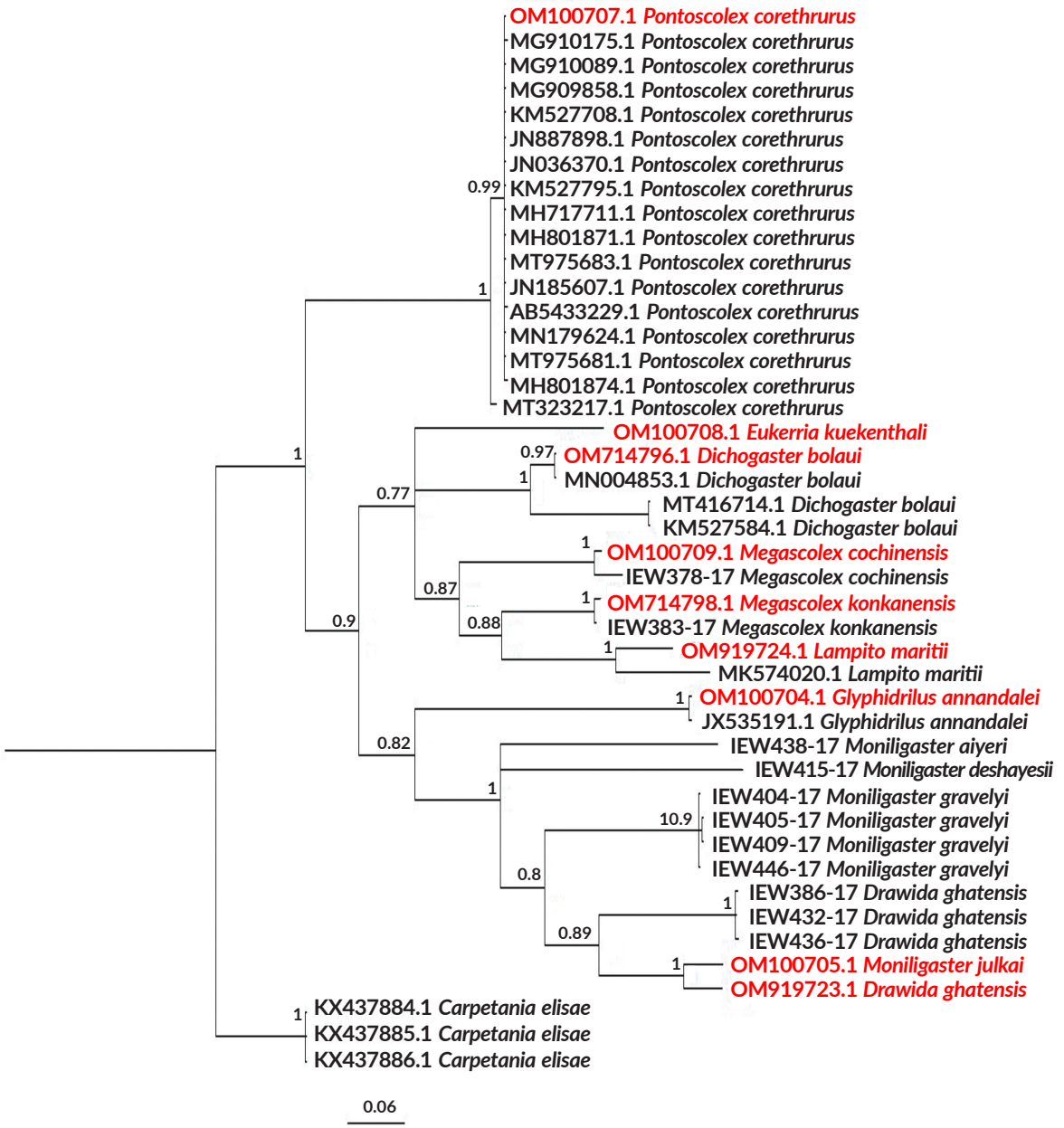

Fig. 11. Bayesian phylogram of 44 earthworm CO1 sequences with *Carpetania elisae* as out–group.

Fig. 11. *Filograma bayesiano de 44 secuencias COI de lombrices de tierra con* Carpetanis elisae *como grupo externo.*

L. mauritii was recorded for the first time from Kottayam district (Anuja et al 2023). It is a native peregrine species that exhibits a wide distribution throughout Kerala and also in other states (Narayanan et al 2016a, 2023a). *P. corethrurus* is a naturalized exotic invasive species that has invaded almost all disturbed areas of the state within a time span of 100 years (Narayanan et al 2016b). *D. bolaui* is another exotic species, widely distributed throughout the state (Narayanan et al 2016a). *E. kuekenthali* might have originated in the warmer regions of South America (Blakemore 2012); it recorded for the first time from Kerala, and a third report from mainland India (Anuja et al 2020) forms a new addition to the GenBank barcode database.

The CO1 sequences of the present study are grouped with other conspecific sequences retrieved from NCBI GenBank and BOLD database, confirming their identification. Because *Moniligaster julkai* and *E. kuekenthali* from this study form a new addition to the GenBank barcode database, no conspecific sequences were obtained during the BLAST analysis. *E. kuekenthali* of the present study stands alone because there was no other

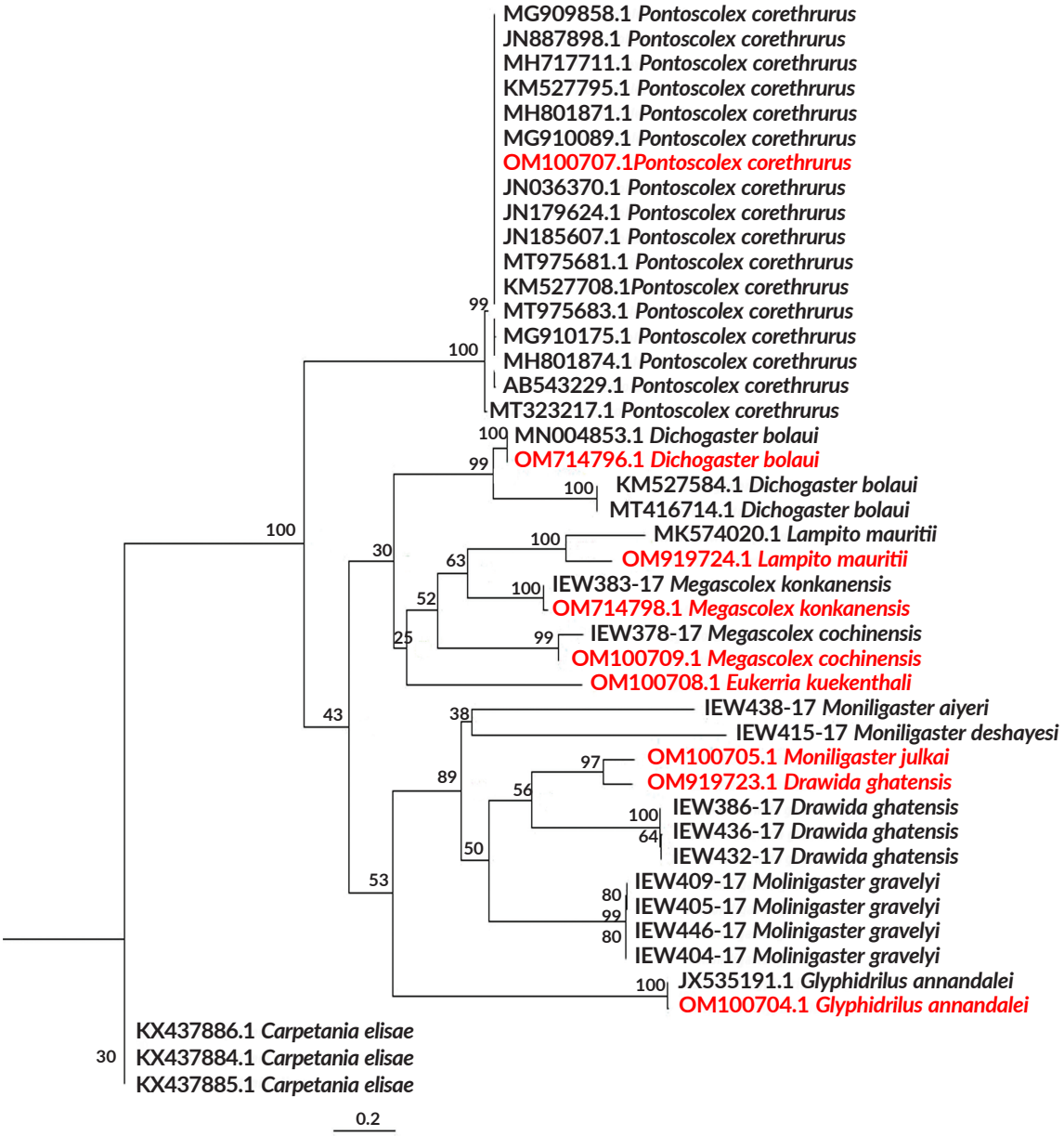

Fig. 12. Maximum likelihood phylogram of 44 earthworm CO1 sequences with Carpetania elisae as out–group.

Fig. 12. *Filograma de máxima probabilidad de 44 secuencias COI de lombrices de tierra con* Carpetanis elisae *como grupo externo.*

conspecific or congeneric sequence in the database. Hence, this mitochondrial sequence is a new contribution to the database of this study.

The exotic species, *P. corethrurus* in the present study shares node with conspecific from India (Tamil Nadu, Northeastern India) and also from other countries like Indonesia, Mexico, Taiwan, Brazil, French Guiana, and Philippines (Decaëns et al 2016, Blakemore 2016, Taheri et al 2018, Potapov et al 2021). No genetic differences were noted between some of the *P. corethrurus* individuals collected from other countries and from the present study. However, genetic variations were observed in other cases, with genetic distances of 0.2 % and 1.7 %. (table 3). *P. corethrurus* is the most widespread earthworm in the tropics (Plisko 2001, Römbke et al 2009) and sub-tropics (Taheri et al 2018). Since *P. corethrurus* is a widespread species it shows considerable intraspecific genetic variation to adapt to different environments (Taheri et al 2018) and the same is also noted in the present study (fig. 11, 12). Future research will be required to distinguish the diversity within the genus *Pontoscolex* (both DNA and morphological). The best way to answer these questions is through genetic comparison with the type specimens of already named species (Taheri et al 2018).

In the present study, *M. julkai* has shown more affinity towards *D. ghatensis* than to other *Moniligaster* species. A genetic distance of approximately 6.9 %

Table 2. Details of COI sequences retrieved from NCBI and BOLD database.

Tabla 2. *Datos de las secuencias del COI extraídos del Centro Nacional de Información Biotecnológica de los Estados Unidos (NCBI) y de la base de datos BOLD.*

No.	Species name	BOLD database	NCBI	Reference
1	*P. corethrurus*	AAF0317	MG910175	BOLD system and NCBI database
2	*P. corethrurus*	AAF0317	MG910089	BOLD system and NCBI database
3	*P. corethrurus*	AAF0317	MG909858	BOLD system and NCBI database
4	*P. corethrurus*	Not available	KM527708	NCBI database
5	*P. corethrurus*	AAF0317	JN887898	BOLD system and NCBI database
6	*P. corethrurus*	AAF0317	JN036370	BOLD system and NCBI database
7	*P. corethrurus*	Not available	KM527795	NCBI database
8	*P. corethrurus*	Not available	MH717711	NCBI database
9	*P. corethrurus*	Not available	MH801871	NCBI database
10	*P. corethrurus*	AAF0317	MT975683	BOLD system and NCBI database
11	*P. corethrurus*	AAF0317	JN185607	BOLD system and NCBI database
12	*P. corethrurus*	AAF0317	AB543229	BOLD system and NCBI database
13	*P. corethrurus*	AAF0317	MN179624	BOLD system and NCBI database
14	*P. corethrurus*	AAF0317	MT975681	BOLD system and NCBI database
15	*P. corethrurus*	Not available	MH801874	NCBI database
16	*P. corethrurus*	AAF0317	MT323217	BOLD system and NCBI database
17	*D. bolaui*	AAM1662	MN004853	BOLD system and NCBI database
18	*D. bolaui*	ABA5891	MT416714	BOLD system and NCBI database
19	*D. bolaui*	Not available	KM527584	NCBI database
20	*M. cochinensis*	ADH2818 (Species Voucher IEW378-17)	Not available	BOLD system
21	*M. konkanensis*	ACU5977 (Species Voucher IEW383-17)	Not available	BOLD system
22	*L. mauritii*	ACV0758	MK574020	BOLD system and NCBI database
23	*G. annandalei*	ACQ2460	JX535191	BOLD system and NCBI database
24	*M. aiyeri*	ADH1655 (Species Voucher: IEW438-17)	Not available	BOLD system
25	*D. deshayesi*	ADH1656 (Species Voucher: IEW415-17)	Not available	BOLD system
26	*D. gravelyi*	ADH0515 (Species Voucher: IEW404-17)	Not available	BOLD system
27	*D. gravelyi*	ADH0515 (Species Voucher: IEW405-17)	Not available	BOLD system
28	*D. gravelyi*	ADH0515 (Species Voucher: IEW409-17)	Not available	BOLD system
29	*D. gravelyi*	ADH0515 (Species Voucher: IEW446-17)	Not available	BOLD system
30	*D. ghatensis*	ADH1313 (Species Voucher: IEW386-17)	Not available	BOLD system
31	*D. ghatensis*	ADH1313 (Species Voucher: IEW432-17)	Not available	BOLD system
32	*D. ghatensis*	ADH1313 (Species Voucher: IEW436-17)	Not available	BOLD system
33	*C. elisae*	ADK1964	KX437884	BOLD system and NCBI database
34	*C. elisae*	ADK1964	KX437885	BOLD system and NCBI database
35	*C. elisae*	ADK1964	KX437886	BOLD system and NCBI database

was obtained between *D. ghatensis* and *M. julkai*, which means that, 6.9% of the base pairs in the genetic marker being compared (COI gene) are different between these two species (table 3). Such a level of genetic distance is typically significant and suggests that the two earthworm species are indeed distinct from each other. This is consistent with their morphological differences, supporting the idea that they are separate species. However, more affinity between these species than to other *Moniligaster* species may be due to *Moniligaster* being polyphyletic in origin. Similarly, *D. ghatensis* is a complex species (Stephenson 1923, Narayanan et al 2023a).

M. konkanensis and *M. cochinensis* in the present study shares nodes with conspecifics from India (Lone et al 2022). However, Lone et al's (2022) descriptions of the above mentioned species have some inconsistencies from the original description (see Narayanan et al 2023a), but here in the present study it shows similarities with Lone et al's (2022) specimens. Hence, we believe thatmolecular wise, Lone et al (2022) identification was correct, but some unintentional error

Table 3. Evolutionary divergence between sequences in percentage (pairwise genetic distance between species).

Tabla 3. *Porcentaje de divergencia evolutiva entre las secuencias (distancia genética por parejas entre especies).*

No.	Species	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44
1	OM100705.1 *M. julkai*																																												
2	IEW438-17 *M. aiyeri*	19.3																																												
3	IEW415-17 *M. deshayesi*	21.4	22.9																																										
4	IEW404-17 *M. gravelyi*	17.6	19.5	20.8																																									
5	IEW405-17 *M. gravelyi*	17.9	19.3	21.0	0.2																																								
6	IEW409-17 *M. gravelyi*	17.9	19.3	21.0	0.2	0.0																																						
7	IEW446-17 *M. gravelyi*	17.6	19.5	20.8	0.0	0.2	0.2																																					
8	OM919723.1 *D. ghatensis*	6.9	19.3	21.6	18.1	18.3	18.3	18.1																																				
9	IEW386-17 *D. ghatensis*	14.7	18.9	21.0	17.6	17.9	17.9	17.6	14.7																																			
10	IEW432-17 *D. ghatensis*	14.9	18.9	20.8	17.4	17.6	17.6	17.4	14.9	0.2																																		
11	IEW436-17 *D. ghatensis*	15.1	18.9	20.8	17.6	17.9	17.9	17.6	15.1	0.4	0.2																																	
12	OM100704.1 *G. annandalei*	22.9	22.1	23.7	21.2	21.4	21.4	21.2	20.4	20.8	20.8	20.8																																
13	JX535191.1 *G. annandalei*	22.7	22.1	23.9	21.0	21.2	21.2	21.0	20.2	20.6	20.6	20.6	0.2																															
14	OM100707.1 *P. corethrurus*	23.5	24.2	23.5	22.9	23.1	23.1	22.9	23.3	22.1	22.1	22.3	23.1	22.9																														
15	MG910175.1 *P. corethrurus*	23.7	24.4	23.7	23.1	23.3	23.3	23.1	23.5	22.3	22.3	22.5	23.3	23.1	0.2																													
16	MG910089.1 *P. corethrurus*	23.5	24.2	23.5	22.9	23.1	23.1	22.9	23.3	22.1	22.1	22.3	23.1	22.9	0.0	0.2																												
17	MG909858.1 *P. corethrurus*	23.5	24.2	23.5	22.9	23.1	23.1	22.9	23.3	22.1	22.1	22.3	23.1	22.9	0.0	0.2	0.0																												
18	KM527708.1 *P. corethrurus*	23.5	24.2	23.5	22.9	23.1	23.1	22.9	23.3	22.1	22.1	22.3	23.1	22.9	0.0	0.2	0.0	0.0																											
19	JN887898.1 *P. corethrurus*	23.5	24.2	23.5	22.9	23.1	23.1	22.9	23.3	22.1	22.1	22.3	23.1	22.9	0.0	0.2	0.0	0.0	0.0																										
20	JN036370.1 *P. corethrurus*	23.5	24.2	23.5	22.9	23.1	23.1	22.9	23.3	22.1	22.1	22.3	23.1	22.9	0.0	0.2	0.0	0.0	0.0	0																								
21	KM527795.1 *P. corethrurus*	23.5	24.2	23.5	22.9	23.1	23.1	22.9	23.3	22.1	22.1	22.3	23.1	22.9	0.0	0.2	0.0	0.0	0.0	0	0																							
22	MH717711.1 *P. corethrurus*	23.5	24.2	23.5	22.9	23.1	23.1	22.9	23.3	22.1	22.1	22.3	23.1	22.9	0.0	0.2	0.0	0.0	0.0	0	0	0																						
23	MH801871.1 *P. corethrurus*	23.5	24.2	23.5	22.9	23.1	23.1	22.9	23.3	22.1	22.1	22.3	23.1	22.9	0.0	0.2	0.0	0.0	0.0	0	0	0	0																					
24	MT975683.1 *P. corethrurus*	23.5	24.2	23.5	22.9	23.1	23.1	22.9	23.3	22.1	22.1	22.3	23.1	22.9	0.0	0.2	0.0	0.0	0.0	0	0	0	0	0																				
25	JN185607.1 *P. corethrurus*	23.5	24.2	23.5	22.9	23.1	23.1	22.9	23.3	22.1	22.1	22.3	23.1	22.9	0.0	0.2	0.0	0.0	0.0	0	0	0	0	0	0																			
26	AB543229.1 *P. corethrurus*	23.5	24.2	23.7	22.9	23.1	23.1	22.9	23.3	22.1	22.1	22.3	23.1	22.9	0.2	0.4	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2																	
27	MN179624.1 *P. corethrurus*	23.5	24.2	23.5	22.9	23.1	23.1	22.9	23.3	22.1	22.1	22.3	23.1	22.9	0.0	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.2															
28	MT323217.1 *P. corethrurus*	23.3	23.9	22.9	22.3	22.5	22.5	22.3	23.1	22.3	22.1	22.3	23.1	23.1	1.7	1.9	1.7	1.7	1.7	1.7	1.7	1.7	1.7	1.7	1.7	1.7	1.9	1.7															
29	MT975681.1 *P. corethrurus*	23.5	24.2	23.5	22.9	23.1	23.1	22.9	23.3	22.1	22.1	22.3	23.1	22.9	0.0	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.2	0.0	1.7													
30	MH801874.1 *P. corethrurus*	23.7	24.4	23.7	23.1	23.3	23.3	23.1	23.5	22.3	22.3	22.5	23.1	22.9	0.2	0.4	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.4	0.2	1.9	0.2											
31	OM100708.1 *E. kuekenthali*	22.9	23.1	22.1	21.8	22.1	22.1	21.8	21.6	22.9	23.1	23.1	19.7	20.0	19.7	20.0	19.7	19.7	19.7	19.7	19.7	19.7	19.7	19.7	19.7	19.7	20.0	19.7	20.0	19.7	20.0										
32	OM714796.1 *D. bolaui*	21.6	21.0	21.2	21.4	21.6	21.6	21.4	21.2	20.4	20.6	20.4	20.6	20.6	21.6	21.8	21.6	21.6	21.6	21.6	21.6	21.6	21.6	21.6	21.6	21.6	21.8	21.6	21.6	21.6	21.8	17.6									
33	MN004853.1 *D. bolaui*	21.6	21.0	21.2	21.4	21.6	21.6	21.4	21.2	20.4	20.6	20.4	20.6	20.6	21.6	21.8	21.6	21.6	21.6	21.6	21.6	21.6	21.6	21.6	21.6	21.6	21.8	21.6	21.6	21.6	21.8	17.6	0.0								
34	MT416714.1 *D. bolaui*	25.8	23.1	21.2	24.6	24.4	24.4	24.6	24.4	20.6	20.4	20.2	22.3	22.3	23.3	23.5	23.3	23.3	23.3	23.3	23.3	23.3	23.3	23.3	23.3	23.3	23.5	23.3	22.5	23.3	23.5	21.2	10.5	10.5							
35	KM527584.1 *D. bolaui*	25.8	23.1	21.2	24.6	24.4	24.4	24.6	24.4	20.6	20.4	20.2	22.3	22.3	23.3	23.5	23.3	23.3	23.3	23.3	23.3	23.3	23.3	23.3	23.3	23.3	23.5	23.3	22.5	23.3	23.5	21.2	10.5	10.5	0.0						
36	OM100709.1 *M. cochinensis*	20.6	21.4	22.9	23.3	23.5	23.5	23.3	19.1	20.2	20.4	20.4	21.4	21.4	19.5	19.7	19.5	19.5	19.5	19.5	19.5	19.5	19.5	19.5	19.5	19.5	19.7	19.5	18.9	19.5	19.7	18.7	16.8	16.8	18.5	18.5					
37	IEW378-17 *M. cochinensis*	20.0	21.4	24.6	23.9	24.2	24.2	23.9	19.1	20.2	20.4	20.4	21.2	21.2	20.2	20.4	20.2	20.2	20.2	20.2	20.2	20.2	20.2	20.2	20.2	20.2	20.2	20.2	19.3	20.2	20.4	19.5	18.9	18.9	18.9	18.9	3.2				
38	OM714798.1 *M. konkanensis*	22.9	21.8	24.4	21.6	21.8	21.8	21.6	24.4	23.7	23.9	24.2	21.6	21.6	20.0	20.2	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.2	20.0	19.5	20.0	20.2	16.6	16.6	16.6	20.2	20.2	16.2	16.2			
39	IEW383-17 *M. konkanensis*	22.5	21.8	23.9	21.2	21.4	21.4	21.2	23.9	23.3	23.5	23.7	21.6	21.6	19.7	20.0	19.7	19.7	19.7	19.7	19.7	19.7	19.7	19.7	19.7	19.7	20.0	19.7	19.3	19.7	20.0	16.4	16.4	16.4	20.0	20.0	16.0	16.0	0.6		
40	OM919724.1 *L.mauritii*	21.4	24.6	22.7	21.4	21.6	21.6	21.4	22.7	23.1	23.3	23.5	23.1	22.9	19.7	20.0	19.7	19.7	19.7	19.7	19.7	19.7	19.7	19.7	19.7	19.7	19.7	19.7	19.3	19.7	20.0	18.3	17.6	17.6	18.9	18.9	16.6	17.6	15.1	14.9		
41	MK574020.1 *L.mauritii*	21.4	24.6	23.3	19.7	20.0	20.0	19.7	22.5	22.3	22.5	22.7	24.4	24.6	21.4	21.6	21.4	21.4	21.4	21.4	21.4	21.4	21.4	21.4	21.4	21.4	21.6	21.4	21.2	21.4	21.6	19.5	17.2	17.2	18.9	18.9	17.2	17.9	16.6	16.0	11.3	0.0	
42	KX437884.1 *C. elisae*	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0	
43	KX437885.1 *C. elisae*	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0	0
44	KX437886.1 *C. elisae*	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0	0

appeared in the species description provided. A genetic distance of 3.2 % was noted between *M. cochinensis*, likely reflecting geographic isolation between the populations (table 3). In the case of *M. konkanensis*, the observed genetic distance 0.6 % reflects typical intraspecific variation, which can arise due to geographic separation, local adaptations, or genetic drift (table 3). It indicates that the individuals are genetically similar but have some minor differences.

G. annandaleim in this study showed the greatest similarity with its conspecific Indian sequences. A genetic distance of 0.2 % between *G. annandalei* is a very small difference, suggesting that the two species are genetically very similar (table 3). Even though the *L. mauritii* that we deposited showed similarity with the sequence retrieved from the database (unpublished) divergence among the clade is high. The 11.3 % genetic distance between them suggests that the two species have been separated for a significant evolutionary period (table 3). This separation has allowed enough time for substantial genetic differences to accumulate, which may reflect adaptation to different environments or other evolutionary pressures. Since it is a native peregrine species, originating from India, and thus showing wide distribution, it may have some cryptic lineages. Because the sample size in this study was very limited, further studies are needed for clarification. The *D. bolaui* oin the present study shares node with conspecificsfrom Kerala (Soumya 2019), Mizoram (Lalthanzara et al 2020) and French Guiana (Decaëns et al 2016). However, *D. bolaui* formed two distinctly separate clades, and this may be due to the presence of cryptic species. Our present results strongly support the usefulness of DNA barcodes in the identification of species of earthworms. The lack of sufficient database references on certain earthworm species in NCBI posed limitations that made it challenging to match the sequence of unknown specimen to known species.

Conclusion

This study is the first comprehensive examination of earthworms in the Kottayam district using an integrated approach that combines molecular and classical taxonomy. It marks the initial investigation of DNA barcoding for earthworms in this district. The manuscript provides the first genomic signatures of two species, *M. julkai* and *E. kuekenthali*, both of which have now been added to the GenBank barcode database.

Future studies should include larger sample sizes, encompass greater taxonomic diversity, and extend their geographic coverage. DNA barcoding allows for the simple incorporation of new taxa into the profile dataset, thereby expanding taxonomic coverage of thedatabase. Moreover, as more taxa are integrated into the dataset, the likelihood of identifying unknown species increases. This research paves the way for the development of a DNA-based identification system for earthworms in laboratories equipped with DNA sequencing facilities. Sustaining a robust and continually updated database is crucial for facilitating the identification and comparison of species worldwide. Additionally, it aids researchers in identifying and studying cryptic and polymorphic forms. It is important to note that relying solely on traditional taxonomical studies based on morphological and anatomical characteristics cannot capture the minute variations that occur at the DNA level. Therefore, it is essential to harness the potential of molecular-level applications in conjunction with traditional taxonomical studies in order to unravel the complexities of evolutionary research. Given that earthworms are regarded as bioindicators of soil changes, studies such as the present study serve as a foundation for future investigations in this field.

References

Andersen JM, Ingram JS, 1993. *Tropical Soil Biology and Fertility. A Handbook of Methods.* CAB International, United Kingdom.

Anuja R, Narayanan SP, Sathrumithra S, Thomas AP, Julka JM, 2020. First Record of Exotic Earthworm *Eukerria kuekenthali* (Michaelsen, 1908) (Annelida: Oligochaeta) from Kerala, India. *Journal of the Bombay Natural History Society (JBNHS)* 117, 160-16. DOI: 10.17087/jbnhs/2020/v117/131225

Anuja R, Narayanan SP, Sathrumithra S, Thomas AP, Julka JM, 2023. Diversity of Earthworms in different land use systems of Kottayam district, Kerala, India. *Proceedings of the National Academy of Sciences, India Section B: Biological Sciences* 93(1), 27-44. DOI: 10.1007/s40011-022-01397-5

Barres L, Sanmartín I, Anderson CL, Susanna A, Buerki S, Galbany-Casals M, Vilatersana R, 2013. Reconstructing the evolution and biogeographic history of tribe Cardueae (Compositae). *American Journal of Botany* 100(5), 867-882. DOI: 10.3732/ajb.1200058

Blakemore RJ, 2012. *Cosmopolitan earthworms: an eco-taxonomic guide to the peregrine species of the World*, 5th Edition. VermEcology, Yokohama, Japan.

Blakemore RJ, 2016. Darwin's earthworms (Annelida, Oligochaeta, Megadrilacea) with review of cosmopolitan *Metaphire peguana*–species group from Philippines. *Opuscula Zoologica Budapest* 47(1), 9-30. DOI: 10.18348/opzool.2016.1.9

Brown WM, George JrM, Wilson AC, 1979. Rapid evolution of animal mitochondrial DNA. *Proceedings of the National Academy of Sciences* 76(4), 1967-1971. DOI: 10.1073/pnas.76.4.1967

Chang CH, James S, 2011. A critique of earthworm molecular phylogenetics. *Pedobiologia* 54, S3-S9. DOI: 10.1016/j.pedobi.2011.07.015

Csuzdi C, Zicsi A, 2003. Earthworms of Hungary (Annelida: Oligochaeta, Lumbr*icidae) Budap*est: *Hungarian Natural History Museum* 271. DOI: 10.5281/zenodo.4309820

Decaëns T, Porco D, James SW, Brown GG, Chassany V, Dubs F, Dupont L, Lapied E, Rougerie R, Rossi JP, Roy V, 2016. DNA barcoding reveals diversity patterns of earthworm communities in remote tropical forests of French Guiana. *Soil Biology and Biochemistry* 92, 171-183. DOI: 10.1016/j.soilbio.2015.10.009

Deepthi MP, Kathireswari P, 2016. Earthworm diversity and analysis of soil inhabited by earthworms in the Vatakara area, Kozhikode, Kerala, India. *International Journal of Current Microbiology and Applied Science* 5(3), 917-925. DOI: 10.20546/ijcmas.2016.503.106

Gates GE, 1972. *Burmese earthworms: an introduction to the systematics and biology of megadrile oligochaetes with special reference to Southeast Asia. Transactions of the American Philosophical Society* 62(7), 1-326. DOI: 10.2307/1006214

Giraddi, RS, Kale RD, Biradar DP, 2014. Earthworms and organic matter recycling-an overview from Indian perspective. *Karnataka Journal of Agricultural Sciences* 27(3), 273-284.

GoK, 2003. *Tenth Five Year Plan 2002-2007: Report of the Steering Committee on Water Resources* (Thiruvananthapuram: State Planning Board, Government of Kerala).

Hall TA, 1999. *BioEdit: A User-Friendly Biological Sequence Alignment Editor and Analysis Program for Windows 95/98/NT.* Nucleic Acids Symposium Series 41, 95-98.

Hebert PD, Gregory TR, 2005. The promise of DNA barcoding for taxonomy. *Systematic Biology* 54(5), 852-859. DOI: 10.1080/10635150500354886

Hijam SD, Thounaojam RS, Kalita MC, Talukdar NC, 2022. Identification of selected earthworm species of Northeast India using DNA barcoding. *Journal of Environmental Biology* 43(5), 716-721. DOI: DOI: 10.22438/jeb/43/5/MRN-1976

James SW, Porco D, Decaëns T, Richard B, Rougerie R, Erseus C, 2010. DNA barcoding reveals cryptic diversity in *Lumbricus terrestris* L., 1758 (Clitellata): resurrection of *L. herculeus* (Savigny, 1826). *PloS*

One 5(12), e15629. DOI: 10.1371/journal.pone.0015629
Julka JM, 1988. *The Fauna of India and the Adjacent Countries: Megadrile Oligochaeta (earthworms). Haplotazida, Lumbricina. Megascolecoidea, Octochaetidae*. Zoological Survey of India.
Karthigeyan M, Mani P, Alagesan P, 2019. Collection, molecular identification and study of earthworm species procured from different soil habitats of Madurai district, Tamil Nadu, India. *Pharmaceutical Innovations* 8, 34-39.
King RA, Tibble AL, Symondson WO, 2008. Opening a can of worms: unprecedented sympatric cryptic diversity within British lumbricid earthworms. *Molecular Ecology* 17(21), 4684-4698. DOI: 10.1111/j.1365-294x.2008.03931.x
Kumar KS, Kumar US, Neelanarayanan P, 2013. Mitochondrial 16S RNA sequence for earthworm identification from Semi Evergreen Forest type of Puliyancholai Reserved Forest in Kolli Hill, Tamil Nadu, south India. *Journal of Microbiology and Biotechnology Research* 3(1), 40-45.
Kumar S, Stecher G, Li M, Knyaz C, Tamura K, 2018. MEGA X: molecular evolutionary genetics analysis across computing platforms. *Molecular biology and evolution* 35(6), 1547.
Kumari S, Kumari C, Narayanan SP, Dev K, Julka JM, 2021a. Phylogenetic relatedness of *Drawida japonica* (Michaelsen, 1892) and *Drawida nepalensis* Michaelsen, 1907 (Clitellata: Moniligastridae) from the Western Himalaya. India. *Annals of Biology* 37(1), 36-41.
Kumari S, Narayanan SP, Julka JM, 2021b. DNA barcoding of *Aporrectodea caliginosa* species-complex earthworms revealed the presence of *Aporrectodea tuberculata* (eisen, 1874) and *Aporrectodea trapezoides* (Dugés, 1828) from the Western Himalaya, India. *Annals of Agricultural Bio Research* 26(1), 20-24.
Kushwaha T, Vishwakarma A, Paliwal R, Burla S, Yadav S, 2015. A Simple Protocol to extract DNA from earthworm tissue for Molecular studies. Research & Reviews. *Journal of Zoological Sciences* 4, 33-37.
Lalthanzara H, Lalfelpuii R, Zothansanga C, Vabeiryureilai M, Kumar NS, Gurusubramanium G, 2018. Oligochaete taxonomy: the rise of earthworm DNA barcode in India. *Science Vision 18*(1), 1-10.
Lalthanzara H, Zothansanga C, Lalchhanhima M, Kumar NS, Ngukir J, Kimsing A, Vabeiryureilai M, 2020. Diversity and new records of earthworms in Arunachal Pradesh, Northeast India. *Journal of Environmental Biology* 41(4), 874-883. DOI: 10.22438/jeb/4(SI)/MS_1921
Lone AR, Thakur SS, Tiwari P, James SW, Yadav S, 2022. Phylogenetic Relationships in earthworm *Megascolex* species (Oligochaeta: Megascolecidae) with addition of two new species. *Diversity* 14(11), 1006. DOI: 10.3390/d14111006
Lone AR, Tiwari N, Thakur SS, Pearlson O, Pavlíček T, Yadav S, 2020. Exploration of four new *Kanchuria* sp. of earthworms (Oligochaeta: Megascolecidae) from the North Eastern Region of India using DNA bar-coding approach. *Journal of Asia-Pacific Biodiversity* 13(2), 268-281. DOI: 10.1016/j.japb.2020.02.004
Marchán DF, Fernández R, de Sosa I, Cosín DJD, Novo M, 2017. Pinpointing cryptic borders: Fine-scale phylogeography and genetic landscape analysis of the *Hormogaster elisae* complex (Oligochaeta, Hormogastridae). *Molecular Phylogenetics and Evolution* 112, 185-193. DOI: 10.1016/j.ympev.2017.05.005
Mathur A, Bhat R, Verma SK, Prakash A, Prasad GB, KS Dua VK, 2010. Standardization of method for Genomic DNA extraction in *Eudrilus eugeniae*. *The Indian Pharmacists* 9, 49-51.
Michaelsen W, 1910. Die Oligochäten fauna der vorderindisch-ceylonischen region. AbdruckVerhandelingen der Naturwissenschaftlichen Vereins zu Hamburg 19(5), 1-108.
Mindell D, Sorensen M, Huddleston CJ, Miranda H, Knight A, Sawchuk S, Yuri T, 1997. Phylogenetic relationships among and within select avian orders based on mitochondrial DNA. In: *Avian Molecular Evolution and Systematics*, *Chapter 8:* 211-247 (DP Mindell Ed). Academic Press, California, USA.
Misirlioğlu M, Reynolds J, Stojanović M, Trakić T, Sekulić J, James S, Csuzdi C, Decaëns T, Lapied E, Phillips H, Cameron E, Brown G, 2023. Earthworms (Clitellata, Megadrili) of the world: an updated checklist of valid species and families, with notes on their distribution. *Zootaxa* 5255 (1), 417-438. DOI: 10.11646/zootaxa.5255.1.33
Moore WS, 1995. Inferring phylogenies from mtDNA variation: mitochondrial-gene trees versus nuclear-gene trees. *Evolution* 49(4), 718-726. DOI: 10.1111/j.1558-5646.1995.tb02308.x
Narayanan SP, Anuja R, Ambattu PT, Paliwal R, 2022. A new species of *Moniligaster* Perrier, 1872 (Annelida, Moniligastridae) from India, with status revision of *M. deshayesi minor* Michaelsen, 1913. *Opuscula Zoologica Budapest* 53(1), 31-50. DOI: 10.18348/opzool.2022.1.31
Narayanan SP, Paliwal R, Kurien VT, Thomas AP, Julka JM, 2023a. *Earthworms (Clitellata: Moniligastrida, Crassiclitellata) of India: distribution and status*. Department of Printing and Publishing, Mahatma Gandhi University, Kottayam.
Narayanan SP, Kurien V, Anuja R, Hasyagar V, 2023b. Earthworm (Clitellata, Megadrili) fauna of Kuttanad wetland, southern part of Vembanad-Kol Ramsar site, India. *Opuscula Zoologica Budapest* 54, 3-21. DOI: 10.18348/opzool.2023.1.3
Narayanan SP, Paliwal R, Kumari S, Ahmed S, Thomas AP, Julka JM, 2020. Annelida: Oligochaeta. In: *Faunal Diversity of Biogeographic Zones of India: Western Ghats*: 87-102 (K Chandra Ed). Zoological Survey of India, Kolkata.
Narayanan SP, Sathrumithra S, Anuja R, Christopher G, Thomas AP, Julka JM, 2021. Three new species and four new species records of earthworms of the genus *Moniligaster* Perrier, 1872 (Clitellata: Moniligastridae) from Kerala region of the Western Ghats Biodiversity Hotspot, India. *Zootaxa* 4949(2), 381-397. DOI: 10.11646/zootaxa.4949.2.11
Narayanan SP, Sathrumithra S, Christopher G, Thomas AP, Julka JM, 2016a. Checklist of the earthworms (Oligochaeta) of Kerala, a constituent of Western Ghats biodiversity hotspot, India. *Zootaxa* 4193(1), 117-137. DOI: 10.11646/zootaxa.4193.1.5
Narayanan SP, Sathrumithra S, Christopher G, Thomas AP, Julka JM, 2016b. Current distribution of the invasive earthworm *Pontoscolex corethrurus* (Müller, 1857) after a century of its first report from Kerala state, India. *Opuscula Zoologica Budapest* 47(1), 101-107. DOI: 10.18348/opzool.2016.1.101
Narayanan SP, Sathrumithra S, Kuriakose D, Christopher G, Thomas AP, Julka JM, 2014. Earthworms (Oligochaeta: Megadrile) from the Mahatma Gandhi University campus, Kottayam, Kerala. *Malabar Trogon* 12(1-3), 2-9.
Narayanan SP, Paliwal R, Thomas B, Thomas AP, Julka JM, 2024. Three new additions to the earthworm (Clitellata: Megadrili) fauna of Kerala stated from the Western Ghats biodiversity hotspot, south-western India. *Opuscula Zoologica Budapest* 55, 47-57. DOI: 10.18348/opzool.2024.2.47
Phillips HRP, Guerra CA, Bartz MLC, Briones MJI, Brown G, Crowther TW, Ferlian O, Gongalsky KB, van den Hoogen J, Krebs J, Orgiazzi A, Routh D, Schwarz B, Bach EM, Bennett J, Brose U, Decaëns T, König-Ries B, Loreau M, Mathieu J, Mulder C, van der Putten WH, Ramirez KS, Rillig MC, Russell D, Rutgers M, Thakur MP, de Vries FT, Wall DH, Wardle DA, Arai M, Ayuke FO, Baker GH, Beauséjour R, Bedano JC, Birkhofer K, Blanchart E, Blossey B, Bolger T, Bradley RL, Callaham MA, Capowiez Y, Caulfield ME, Choi A, Crotty FV, Dávalos A, Cosin DJD, Dominguez A, Duhour AE, van Eekeren N, Emmerling C, Falco LB, Fernández R, Fonte SJ, Fragoso C, Franco ALC, Fugère M, Fusilero AT, Gholami S, Gundale MJ, López MG, Hackenberger DK, Hernández LM, Hishi T, Holdsworth AR, Holmstrup M, Hopfensperger KN, Lwanga EH, Huhta V, Hurisso TT, Iannone BV 3rd, Iordache M, Joschko M, Kaneko N, Kanianska R, Keith AM, Kelly CA, Kernecker ML, Klaminder J, Koné AW, Kooch Y, Kukkonen ST, Lalthanzara H, Lammel DR, Lebedev IM, Li Y, Lidon JBJ, Lincoln NK, Loss SR, Marichal R, Matula R, Moos JH, Moreno G, Morón-Ríos A, Muys B, Neirynck J, Norgrove L, Novo M, Nuutinen V, Nuzzo V, Rahman P M, Pansu J, Paudel S, Pérès G, Pérez-Camacho L, Piñeiro R, Ponge JF, Rashid MI, Rebollo S, Rodeiro-Iglesias J, Rodríguez MÁ, Roth AM, Rousseau GX, Rozen A, Sayad E, van Schaik L, Scharenbroch BC, Schirrmann M, Schmidt O, Schröder B, Seeber J, Shashkov MP, Singh J, Smith SM, Steinwandter M, Talavera JA, Trigo D, Tsukamoto J, de Valença AW, Vanek SJ, Virto I, Wackett AA, Warren MW, Wehr NH, Whalen JK, Wironen MB, Wolters V, Zenkova IV, Zhang W, Cameron EK, Eisenhauer N, 2019. Global distribution of earthworm diversity. *Science* 366(6464), 480-485. DOI: 10.1126/science.aax4851
Plisko JD, 2001. Notes on the occurrence of the introduced earthworm *Pontoscolex corethrurus* (Müller, 1857) in South Africa (Oligochaeta: Glossoscolecidae). *African Invertebrates* 42(1), 323-334.
Pop AA, Wink M, Pop VV, 2003. Use of 18S, 16S rDNA and cytochrome c oxidase sequences in earthworm taxonomy (Oligochaeta, Lumbricidae): The 7th International Symposium on Earthworm Ecology, Cardiff, Wales 2002. *Pedobiologia* 47(5-6), 428-433. DOI: 10.1078/0031-4056-00208
Posada D, 2008. jModelTest: phylogenetic model averaging. *Molecular Biology and Evolution 25*(7), 1253-1256. DOI: 10.1093/molbev/msn083
Potapov A, Schaefer I, Jochum M, Widyastuti R, Eisenhauer N, Scheu S, 2021. Oil palm and rubber expansion facilitates earthworm invasion in Indonesia. *Biological Invasions* 23(9), 2783-2795. DOI: 10.1007/s10530-021-02539-y
Rambaut A, 2014. FigTree, ver. 1.4. 2. Program distributed by the author (University of Edinburgh, Edinburgh, Scotland).
Rao RK, Laxmi C, Mushan LC, Shaikh SA, 2014. Molecular Phylogenetic Study of Earthworm *Eudrilus eugeniae*. *International Journal of Advanced Biotechnology and Research* 5(2), 202-207.
Reynolds JW, 1977. The earthworms of Massachusetts (Oligochaeta: Lumbricidae, Megascolecidae and Sparganophilidae). *Megadrilogica* 3(2), 49-54.

Römbke J, Schmidt P, Höfer H, 2009. The earthworm fauna of regenerating forests and anthropogenic habitats in the coastal region of Paraná. *Pesquisa Agropecuaria Brasileira* 44, 1040-1049. DOI: 10.1590/S0100-204X2009000800037

Ronquist F, Teslenko M, Van Der Mark P, Ayres DL, Darling A, Höhna S, Larget B, Liu L, Suchard MA, Huelsenbeck JP, 2012. MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. *Systematic Biology* 61(3), 539-542. DOI: 10.1093/sysbio/sys029

Rougerie R, Decaëns T, Deharveng L, Porco D, James SW, Chang CH, Richard B, Potapov M, Suhardjono Y, Hebert PD, 2009. DNA barcodes for soil animal taxonomy. *Pesquisa Agropecuária Brasileira* 44, 789-802. DOI: 10.1590/S0100-204X2009000800002

Sathrumithra S, Narayanan SP, Anuja R, Kurian P, Thomas AP, Julka JM, Reynolds JW, 2018. Diversity of earthworms (Annelida: Oligochaeta) in Konni, a part of the Western Ghats of Kerala India. *Megadrilogica* 23(3), 57-60.

Schwert DP, 1990. Oligochaeta: Lumbricidae. In: *Soil Biology Guide:* 341-356 (DL Dindal, Ed). John Wiley and Sons, New York.

Shekhovtsov SV, Sundukov YN, Blakemore RJ, Gongalsky KB, 2018. Identifying earthworms (Oligochaeta, Megadrili) of the southern Kuril Islands using DNA barcodes. *Animal Biodiversity and Conservation* 41(1), 9-17. DOI: 10.32800/abc.2018.41.0009

Silvestro D, Michalak I, 2012. raxmlGUI: a graphical front-end for RAxML. *Organisms Diversity and Evolution* 12, 335-337. DOI: 10.1007/s13127-011-0056-0

Soumya L, Poovathingal KR, Williams, GP, Kunnummal SV, 2022. DNA Barcoding of earthworms of Coorg region of Karnataka, India and study of their physico-chemical habitat. *Universal Journal of Agriculture Research* 10(6), 761-784. DOI: 10.13189/ujar.2022.100615

Stephenson J, 1923. *The Fauna of British India Including Ceylon and Burma: Published Under the Authority of the Secretary of State for India in Council. Oligochœta*. Taylor and Francis, London.

Taheri S, James S, Roy V, Decaëns T, Williams BW, Anderson F, Rougerie R, Chang CH, Brown G, Cunha L, Stanton DWG, Dupont L, 2018. Complex taxonomy of the 'brush tail' peregrine earthworm *Pontoscolex corethrurus*. *Molecular Phylogenetics and Evolution* 124, 60-70. DOI: 10.1016/j.ympev.2018.02.021

Thakur SS, Lone AR, Tiwari N, Jain SK, James SW, Yadav S, 2021. A contribution to the earthworm diversity (Clitellata, Moniligastridae) of Kerala, a component of the Western Ghats biodiversity hotspot, India, using integrated taxonomy. *Animal Biodiversity and Conservation* 44(1), 117-137. DOI: 10.32800/ abc.2021.44.0117

Thakur SS, Yadav S, 2018. *Exploration of Earthworms of India through Online Digital Library*. Intechopen. DOI: 10.5772/intechopen.75666

Vabeiryureilai M, Zothansanga C, Lalchhanhima M, Kumar NS, Lalthanzara H, 2020. Study on the *Amynthas* (Kinberg, 1867) earthworm (Megascolecidae: Oligochaeta) diversity through DNA barcoding from Northeast India. *Journal of Environmental Biology* 41(4), 867-873. DOI: 10.22438/jeb/4(SI)/MS_1919

Acknowledgements
The authors are grateful to the colleagues in the department for the help provided during the fieldwork and also to Mr. Naveen Babu for preparing the map. The first author wishes to acknowledge the Kerala State Biodiversity Board, Thiruvananthapuram, for the fellowship provided.

Author contributions
Conceptualization, **AP Thomas**, **R Anuja**, **S Prasanth Narayanan**; logistic facility, **AP Thomas**; investigation and field work, **R Anuja**; formal analysis, **R Anuja**, **B Padmakumar**, **NV Gopika**, **S Kumari**; identification, curation and drawings, **R Anuja**, **S Prasanth Narayanan**; writing-original draft preparation, **R Anuja**; writing-review and editing, **R Anuja**, **S Prasanth Narayanan**, **L Mathew**; supervision, **AP Thomas**, **L Mathew**.

Conflicts of interest
Authors have no conflicts of interest

Funding
The work is partially funded by Kerala State Biodiversity Board, Thiruvananthapuram.

Complete affiliations
R. Anuja, S. Prasanth Narayanan, Babu Padmakumar, N. V. Gopika, A. P. Thomas, Advanced Centre of Environmental Studies and Sustainable Development, Mahatma Gandhi University, Priyadarsini Hills, Kottayam-686560, Kerala, India.
Shailja Kumari, Department of Bio Sciences, Career Point University, Hamirpur- 176045, Himachal Pradesh, India.
Linu Mathew, School of Biosciences, Mahatma Gandhi University, Priyadarsini Hills, Kottayam-686560, Kerala, India.