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New Earthworm Record from Division Muzaffarabad, Azad Kashmir, Pakistan Supported by Molecular Markers

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Abstract

Earthworm diversity and ecology in Pakistan is poorly known, especially in the region of Azad Jammu & Kashmir. An earthworm community survey assisted by genetic barcoding detected an unidentified species which could constitute a new record for Pakistan. Morphological study revealed its identity as *Perelia kaznakovi*. Additionally, Bayesian phylogenetic inference based on five mitochondrial and nuclear molecular markers was performed. Results provided a phylogenetic placement of the genus *Perelia* within Lumbricidae for the first time, indicating a close relationship with *Eophila*. This approach should be implemented to *Perelia arnoldiana* and further representatives of the genus in order to understand their biogeography, diversity and evolutionary history.

Key words: Azad Kashmir, earthworms, Crassiclitellata, *Perelia*, integrative systematics, molecular barcoding

Introduction

Earthworms are one of the most important components of the soil fauna because they regulate the physical, chemical, and biological properties of soil (Lone *et al*. 2020). Within the last ten years, the public interest in soil organisms (particularly earthworms) has significantly increased due to their functional role at agricultural sites (van Groenigen *et al*., 2014). Earthworms are mostly terrestrial organisms, that originated around 250 million years ago in Paleozoic Era, characterized by their segmented body. They belong to the phylum Annelida and class Oligochaeta (Fragoso *et al*. 2003; Lavelle & Spain, 2001). Misirlioðlu et al. (in press, this issue) stated that more than 5,000 species belonging to 23 families are currently known (but their real diversity could be over 8,000).

Lumbricidae Rafinesque-Schmaltz, 1815 is the dominant earthworm family in the Holarctic region, a monophyletic group included in the Crassiclitellata (James & Davidson, 2012). Lumbricidae includes ~42 genera and ~670 species (Blakemore, 2008). The identification of adult earthworm species is based on external and internal morphological features, but molecular phylogenetic tools have been shown usefully complement them (Marchán et al. 2022). Recent molecular phylogenetic analyses have highlighted the need for the revision of current Lumbricidae taxonomy (Pop *et al.* 2003; King *et al.* 2008; Briones *et al*. 2009; Pérez-Losada *et al.* 2009, 2011; Klarica *et al.* 2012; Pérez-Losada et al. 2015; Domínguez *et al.* 2015).

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Although earthworms are well-studied invertebrates all over the world, they are thoroughly neglected organisms in Pakistan. The earthworm fauna of Pakistan has been partially described on the basis of morphological features (Ghafoor & Qureshi, 1999; Andleeb *et al.* 2016) with 31 species reported to this day. Yet, molecular approaches have not been applied in Pakistan for earthworm species identification. For the region of Azad Jammu and Kashmir (AJ&K) almost no data on diversity, distribution and abundance of earthworms exists, with a single reported species – *Eisenia fetida* (Savignyi) by Andleeb *et al*. (2016). Within a wider study exploring the earthworm communities from AJ&K through DNA barcoding (cytochrome c oxidase subunit I sequencing) one of the retrieved species did not match with the species available in existing databases. In this work, the novel earthworm record for Pakistan is studied in detail through morphological and molecular phylogenetics analyses, highlighting the poorly known biodiversity of the studied region.

MATERIALS AND METHODS

Study area and sampling

Muzaffarabad is the capital of Azad Jammu & Kashmir, Pakistan positioned on the banks of the Jhelum and Neelum rivers and located at 34°21′30″N, 73°28′20″E, and an elevation of 737 m above sea level. The soil moisture contents were in the range from 30% to 61%, soil pH was 6.5 to 7.4, and the minimum and maximum air temperatures during July were about 23°C-35°C, and in January 3°C-16°. Earthworms were collected using three different methods such as 0.2-0.5% diluted formalin (Paoletti *et al*. 2013; Raw 1959), digging using quadrate method (25 x 25 cm) and hand-sorting methods (Pelosi *et al.* 2009). Sampling was conducted in 2019 and 2020.

Preservation

After collection, earthworms were placed in cold water for at least 24 h to clean their guts. Washing also removed the impurities and mud on the surface of worms. Some earthworm species were washed with 75% ethanol to remove soil contamination, then tail region was cut and preserved in 100% ethanol for molecular analysis. Remaining body was preserved in 4% formalin and after several days transferred to 100% ethanol for morphological studies. The samples were stored at 4 °C for future work and for permanent preservation, 5.0 % formalin was used (Jalal *et al.* 2014).

Taxonomic evaluation

The external morphological and internal characters were examined under a Nikon SMZ1500 stereomicroscope. Species classification and morphological diagnoses were carried using the set of external and internal morphological characters used by Qiu & Bouché (1998a, 1998b, 1998c, 1998d), and following the format established by Domínguez *et al*. (2018). Main external morphological characters were average length, average number of segments, average weight, pigmentation, type of prostomium, chaetal arrangement, position of papillae, position of first dorsal pore, nephridial pore arrangement, position and development of male pores, position and development of female pores, position of spermathecal pores, position of clitellum, position of tubercula pubertatis. Main internal anatomical characters, i.e., position of esophageal hearts, position and morphology of calciferous glands, position of crop, position of gizzard, type of typhlosole, shape of nephridial bladders, number and position of seminal vesicles, and number and position of spermathecae were also recorded.

Genomic DNA extraction, PCR amplification and sequence analysis

Total genomic DNA was extracted using DNeasy Blood & Tissue Kit (Qiagen) from ventral integument tissue of three specimens identified as *Perelia kaznakovi* (Michaelsen). COI, ND1, 16S, 28S, and 12S primers were used to amplify the regions of the nuclear 28S rRNA and mitochondrial 16S rRNA, 12S rRNA, NADH dehydrogenase

(*ND1*) and cytochrome c oxidase subunit I—(*COI*)) at Torre CACTI lab 97, Department of Ecoloxia, University of Vigo, Spain. The standard protocol established for the International Barcode of Life project (http://ibol.org/) (Decaëns *et al*. 2016) was used for the polymerase chain reaction (PCR), with primers and conditions described in Pérez-Losada *et al.* (2009, 2015). The detail of primer sequences is given in Table 1. The PCR mix contained 4 µL of PCR buffer 10 x plus MgCl2, 0.8 µL dNTP 2 mM, 1 µL Taq DNA polymerase 1U/ µL, 2 µL of each primer, 28.2 µL PCR water and 2 µL of DNA. A final volume of 40 µL was obtained by addition of 38 µL mix and 2 µL sample. PCR amplifications were performed in an Axygen™ MaxyGene™ Gradient Thermal Cycler (Axygen Scientific THERM1001, USA) subjected to: 3 min of denaturation at 94 ˚C, followed by 40 cycles at 94 ˚C for 30 s, annealing at 45 ˚C (COI, ND1), 50 ˚C (16S, 28S) and 55 ˚C (12S) for 45 sec and 1 min at 72 ˚C, followed by a final elongation step of 10 min at 72 ˚C, and hold at 20 ˚C. PCR products were purified and sequenced by the C.A.C.T.I Genomics service (University of Vigo) *via* Sanger sequencing method.

DNA sequences obtained in this study are available in Genbank: accession numbers are OP964794, OP965384-OP965386, OP970561.

Sequence alignment and phylogenetic analysis

Sequences for all the molecular markers from representatives of most of the Lumbricidae genera and two members of the closest families (Hormogastridae and Criodrilidae) were downloaded from Genbank and used as a reference dataset to reconstruct the phylogenetic relationships of *Perelia kaznakovi*.

Sequences were aligned with MAFFT v.7 (Katoh & Standley, 2013) with default settings and concatenated with BioEdit (1999), resulting in a matrix of 4164 bp. The best fitting evolutionary model for each partition was selected with jModelTest v. 2.1.3. (2012) by applying the Akaike information criterion (AIC; Akaike 1973) and the Bayesian information criterion (BIC; Schwarz 1978). GTR + I + G was selected as best-fitting evolutionary model for *COI*, *28S* and *ND1*, GTR + G was selected for *12S*, and HKY+I+G was selected for *16S*.

Bayesian Inference of the phylogeny was estimated for both datasets with MrBayes v.3.2.6 (Ronquist, F. & Huelsenbeck 2003) as implemented in CIPRES Science Gateway V. 3.3 (Miller *et al.* 2010). The analysis was performed with default parameters, and each of the two independent runs was set to 50 million generations sampling every 5,000th generation (10,000 trees). Twenty-percent of the trees were discarded as burn-in, with remaining trees combined and summarized on a 50% majority-rule consensus tree.

RESULTS AND DISCUSSION

Systematics studies

Phylum Annelida (Lamarck, 1802)

Class Oligochaeta (Grube, 1850) / Clitellata (Michaelsen, 1919)

Order Haplotaxida (Michaelsen, 1900)

Family Lumbricidae (Rafinesque-Schmaltz, 1815)

Genus *Perelia* (Easton, 1983)

Perelia kaznakovi (Michaelsen, 1910)

Helodrilus kaznakovi Michaelsen, 1910: 65.
Allolobophora kaznakovi (Michaelsen, 1910)
Eophila asiatica Malevic, 1949: 1005.
Helodrilus (Eophila) kaznakovi: Perel 1976
Perelia kaznakovi: Easton 1983

Distribution and ecology

Perelia kaznakovi is rarely found worldwide (Iran, Kirghizstan, Tajikistan, Turkmenistan and Uzbekistan - Csuzdi and Pavlíček, 2005; Rakhmatullaev *et al*. 2010; Asirovic, 2011; Mirmonsef *et al*. 2011; Farhadi *et al*. 2013), and it is usually associated with very high altitudes and cold and moist habitats. In the current study *Perelia kaznakovi* was found in the moist warm temperate lands of Muzaffarabad i.e. Markaz (Kahori), Union council Balgran (villages; Balgran, Gratnarh, Kelgran, and Manjhoter), with GPS coordinates 34°44′27.5″N 73°68′92.6″ E. *P. kaznakovi* was found in loamy cultivated soils, either alone or in association with other earthworm species such as *Aporrectodea trapezoides* and *Allolobophora chlorotica*.

Diagnostic features and description

Internal and external features are illustrated in Figure 1 and 2.

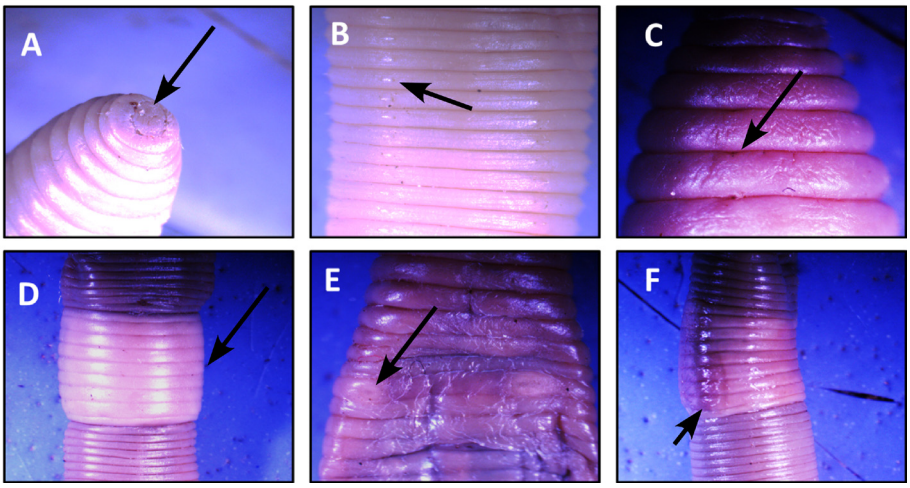

FIGURE 1: External morphological features of *Perelia kaznakovi*. A= prostomium, B= chaetal arrangement, C= dorsal pore, D= clitellum, E= male pore, F= tubercula pubertatis

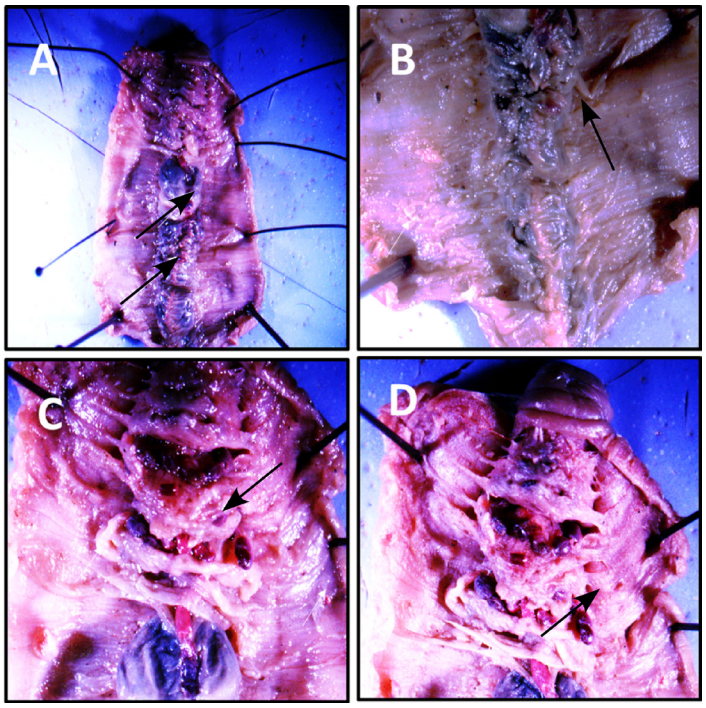

FIGURE 2: Internal anatomy of *Perelia kaznakovi.* A= Gizzard and intestine, B= S-shaped nephridia, C= seminal vesicles, D= spermathecae

Body cylindrical and dorso-ventrally flattened, having blunt anterior and posterior end. Unpigmented with whitish saddle-shaped clitellum, sometimes with brownish color on dorsum. Prostomium epilobic, chaetae closely paired. First dorsal pore on 4/5, spermathecal pores on 10 and 11 in setal line cd. Clitellum in segments 27 to 35, tubercula pubertatis on 31-34. Male pore on 15th, fairly visible, usually with glandular crescent intruding into the neighboring segments, female pore inconspicuous. Well-developed genital papillae around setae *ab* on 10, 11 and 27, 28. (Figure 1). Nephropores irregularly alternate between b and above cd. Two pairs of spermathecae are present on furrow 9/10 and 10/11. Two pairs of seminal vesicles are also present on segment 11th and 12th. Nephridial bladder is S-shaped starting from 4th segment. Longitudinal musculature fasciculate with strong radial walls (Figure 2). Our findings are in agreement with the review of the morphology of *Perelia kaznakovi* by Csuzdi and Pavlíček, (2005). Other species attributed to *Perelia* in recent revisions are *Perelia galileana* Csuzdi and Pavlíček*, Perelia shamsi* Csuzdi and Pavlíček*, Perelia makrisi* Szederjesi, Pavlíček and Csuzdi*, Perelia phoebea (*Cognetti de Martiis)*, Perelia aharonii (*Stephenson)*, Perelia hatayica* Csuzdi, Pavlíček and Misirlioglu, *Perelia nematogena* (Rosa) (Csuzdi and Pavlíček, 2005; Csuzdi *et al.* 2007; Szederjesi *et al*. 2013a; Szederjesi *et al*. 2016).

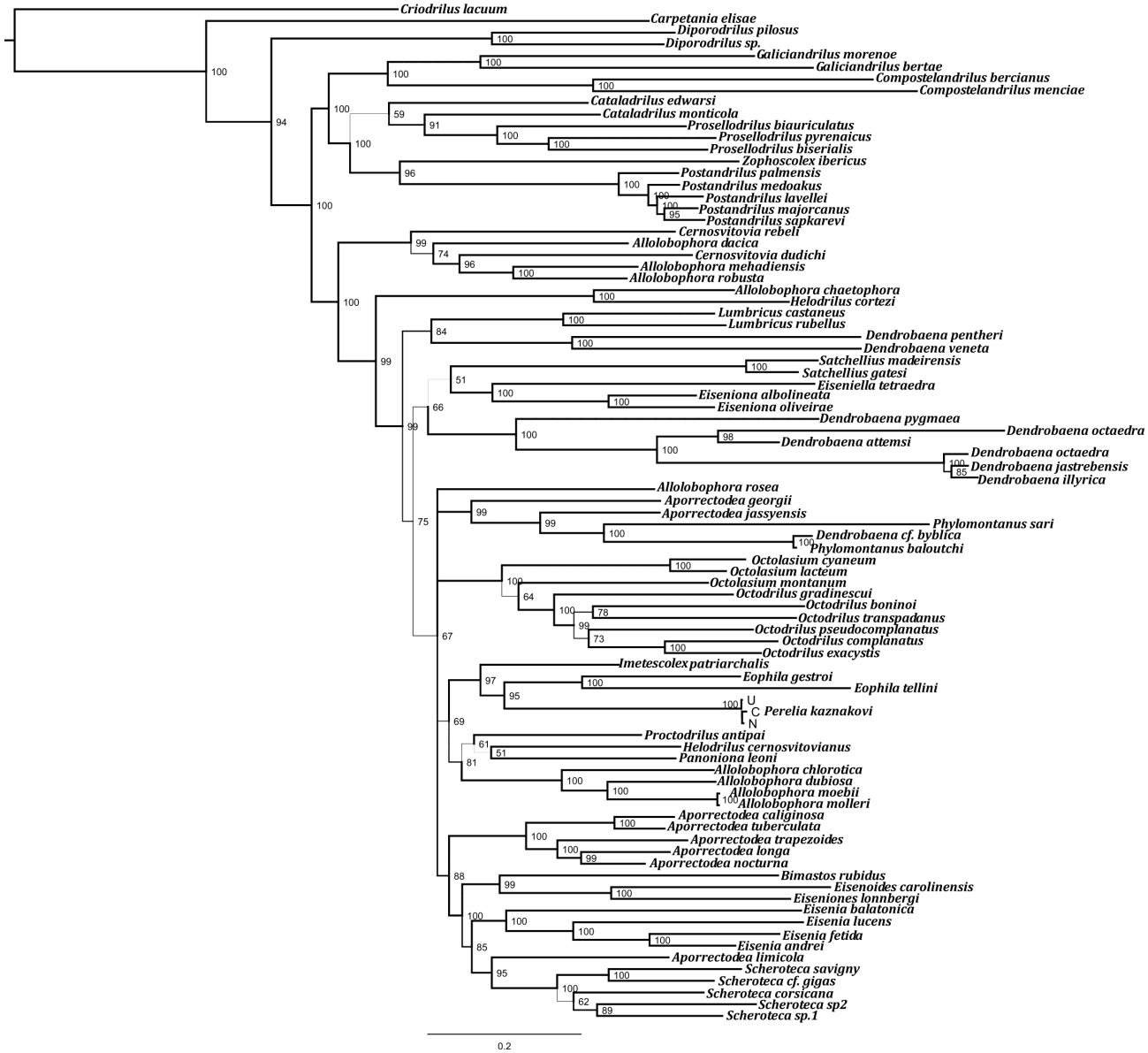

FIGURE 3: Fifty percent majority-rule consensus tree. It shows the phylogenetic relationships of *Perelia kaznakovi* obtained by Bayesian phylogenetic analysis of the concatenated sequence of molecular markers *COI–16S-12S–ND1–28S*. Posterior probability support values are shown beside the corresponding nodes. The bottom bar shows the scale of the branch lengths.

Phylogenetic relationships

The Bayesian phylogenetic tree (Figure 3) recovered *Perelia kaznakovi* as a sister clade to *Eophila* (represented by *Eophila tellinii* and *Eo. gestroi*) with high support (posterior probability = 95%). The clade formed by the latter was recovered as sister to *Imetescolex patriarchalis*, and all of them were include in a weakly supported clade comprising *Helodrilus*, *Proctodrilus* and *Allolobophora*.

The phylogenetic relationships recovered by the analyses are somewhat supported by traditional morphology-based taxonomy: *Eophila gestroi* has been previously assigned to *Perelia* on the basis of their morphology (Blakemore, 2008). Even though the assignment of *Eo. gestroi* to *Eophila* is currently well supported by its close relationship to *Eo. tellinii* (generotype of *Eophila*) as shown by De Sosa et al (2019), molecular phylogenetics suggest a close link between both genera.

This work is the first instance of the inclusion of a representative of *Perelia* in a molecular phylogenetic framework. The relationships of this very diverse genus (30 species) with other Lumbricidae genera are unfortunately poorly known, yet they might hold interesting clues for the evolutionary history and historical biogeography of the family. In order to advance in this topic, it would be necessary to study *Perelia arnoldiana* (Perel) -generotype of *Perelia*- and other representatives across its wide range (from eastern Europe to Central Asia) by molecular phylogenetic analyses.

Conclusions

Integrative systematics allowed identification of *Perelia kaznakovi* specimens from Azad Jammu & Kashmir, extending the eastern limit of its range to Pakistan. Additionally, this methodology provided initial insight into the position of the diverse genus *Perelia* within the evolutionary tree of Lumbricidae.

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