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MORPHO MOLECULAR TAXONOMY AND BIOINFORMATICS BASED PHYLOGENY OF *Metaphire posthuma* (SIMS AND EASTON 1972)

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ABSTRACT

The species of earthworm *Pheretima posthuma* (Vaillant, 1868) now known as *Metaphire posthuma* (Sims and Easton, 1972) collected from Ranchi, has been re-described based on morphological features following the key of Chang *et al*. (2016). The specimen has been further identified on molecular basis for the first time from Jharkhand state. The Ranchi specimen GenBank accession number PP832872 showed genetic difference form specimen collected from Uttar Pradesh, Punjab and Mizoram while it revealed 100% similarity with specimens from Thailand and China. The intra-species phylogeny has been established on the basis of BLAST score when successfully generated DNA barcode sequence of mitochondrial Cytochrome C Oxidase – 1 (CO1) of the specimens was subjected to it. The distance matrix has been calculated to get pair wise genetic difference among the twelve strains of the species obtained from GenBank and our sequence on the basis of BLAST test. The morphology based key appeared to be applicable for this species.

INTRODUCTION

Pheretima posthuma (Vaillant, 1868) presently known as *Metaphire posthuma* (Sims and Easton 1972) is the most common name among Indian earthworms and is part of curriculum of universities as type study not only in India but also in Burma, Sri Lanka, Japan, China and Philippines and is well studied species (Bahl 1919, 1921, 1922, 1924, 1926, 1927, 1934, 1941, 1942, 1947) and the publication of detailed account of anatomy and physiology of the species as Zoological Memoire (Bhal,1926), presented a comprehensive account.

listed and characterized almost all of the Indian species of *Pheretima* inventoried and described by Michaelsen (1909 and 1910) and were recognized as peregrine or of doubtful status, and it was suggested that the dubious names are synonyms of peregrine species. Among the then described species by Michaelsen a single species *anomala*, (Michaelsen, 1909) was considered as "probably endemic" (Gates, 1937)

So far, the origin of *P. posthuma* is considered according to Michaelsen (1909) the species, though doubtfully, is of Burmese or Burmese-Siamese origin and with a south-eastern Asiatic mainland distribution. Such distribution of the species needs no assumption about its anthropogenic distribution (Gates, 1937)

According to Gates (1937) between 1872 to 1937 the inventory of the Indian earthworm species belonging to genus *Pheretima* was a huge one. But after proper examination and re-description many of the species were excluded as mistaken records, eliminated due to being a form probably belonging to other genera which were mistakenly included in the list and a good number of names were reduced due to synonyms. Finally, a total of 13 species were kept belonging to the genus. Even today, the pheretimoid earthworms not only in India, but on global level are widely distributed, have been focal issue. The two genera namely *Amynthas* and *Metaphire*, are of special concernforboth zoologists and ecologistsfrom their proper identification view point, The reason behind such concern is the use of keys for identification which mainly are old one and have almost lost the relevance due to having outdated taxonomic information. These keys are based on more anatomical and less morphological characters (Chang *et al.*, 2016). Taxonomy based on these keys requires precised taxonomic expertise and the specimen needs to be dissected, even from the first step of the key. Such complicated pre-requisites have resulted into an obstacle in broader use of these keys and have posed problem in identification.

On global scale, *Pheretima auct*, the *Pheretima* group of genera, or the *Pheretima* complex are the various terms by which the pheretimoid earthworms are referred to (Sims and Easton 1972; Chang *et al.*, 2009a; 2016; Blakemore 2003, 2010a, 2013a, 2014). In this group previously there was a single genus *Pheretima sensu lato*. But now the pheretimoid group has become the most interesting fields of research on

earthworm diversity. Four new genera have been described during last twenty years (Chang and Chen 2004, 2005; James 2004, 2005, 2009; Chang *et al*., 2008, 2014; Tsai *et al*., 2009, 2010; Blakemore 2010a, 2010b; 2013b; Hong and James 2011, 2013; Shen *et al*., 2013, 2014), and the group currently contains more than 1,000 species in 14 genera (Sims and Easton 1972; James 2004; Blakemore 2006, 2010b, 2013b,2013c). The various species of this group are now recognized as invasive species in temperate and tropical regions worldwide which specially belong to genera *Amynthas, Metaphire, Pheretima, Pithemera and Polypheretima,* (Sims and Easton 1972; Easton 1981; Gates 1982; Chang *et al*., 2009a; Blakemore 2010a).

Earthworms are soil engineers and play a vital role in providing ecosystem services (Srivastava *et al*., 2022) to understand the soil community structure and function and their alteration by earthworms it is important to identify the earthworm community. Earthworm identification is also essential in order to have a continued ecosystem services to determine the state of the system. Earthworm taxonomy has been nightmare (Beddard,1883) and a challenge since beginning due to changed morphs *i.e.* a wide range of morphological variations in characteristics and accordingly their documentation in taxonomic literature.

The paucity of a user-friendly key poses the problem. The comprehensiveThe keys by Reynolds (1978) and Gates (1982) are outdated. The key given by Blakemore (2010a) has become now the primary resource for identifying peregrine species. Since among most of the keys the first identifying character is anatomical which demands the dissection the specimens from the onset of using the keys, Chang *et al*. (2016) have however put forward the key of identification of mature specimens of the 16 species recorded in the US. Which is possible without dissection. Characters of anatomy though included in the key are for confirmation. This new key to identify the North American and north of Mexican specimen have been used here to identify the specimen. The specimen has further been confirmed on molecular basis using bioinformatic tool.

The present communication deals with identification of specimen based on morphological key of Chang *et al*. (2016) to test whether the key is equally applicable for Indian species and then getting it confirmed by molecular taxonomy and bioinformatic tool based phylogeny.

MATERIALS AND METHODS

Sampling

Earthworms were sampled from different places in and around Ranchi by monolith method from an area of 25 X 25 cm during morning hours following Sinha and Srivastava (2001). The earthworms were hand sorted, photographed and geotagged. After sorting worms were separated into different age groups on the basis of length and clitellar development. Earthworms were preserved in 70% ethanol with little amount of glycerine. The collected specimens were used for taxonomic identification and molecular characterization for confirmation of taxonomic identification as well as for establishing the phylogeny. One of the specimens was submitted to K.C. Bose Memorial Natural History Museum in the Department of Zoology, Ranchi University, Ranchi (Voucher No. ZDRU/EW/107/2023).

DNA extraction, PCR amplification and Sequencing

DNA was isolated using a QIAamp, DNA Mini kit (Qiagen, Hilden, Germany) (QIAmp, 2014). Quality was evaluated on 1% agarose gel, a single band of high-molecular weight DNA was observed. Isolated DNA was amplified with cytochrome oxidase subunit I (COI) specific primer (LCO149F and HCO2198R) using Verit® 96 well thermal cycler, which resulted into single discrete PCR amplicon band. The PCR amplicon was bead purified and further subjected to Sanger sequencing. Bi-directional DNA sequencing reaction of PCR amplicon was carried out with specific primer, using BDT v3.1 cycle sequencing kit on Applied Biosystems 3500 Dx genetic analyzer (ThermoFisher, 2024).

Bioinformatic Tools

The nucleotide sequence following the Sanger sequencing of obtained discrete PCR amplicon was compared with nucleotide databases using blastn (nucleotide blast) tool of BLAST (Basic Local Alignment and Search Tool) program based at NCBI (National Center for Biotechnology Information). Blastn compares a nucleotide query with the nucleotide sequences present in various global nucleotide database systems (NCBI, 2024). Blastn was performed with parameters selected as search set: Standard database; Program selection: Highly similar sequences (megablast) are used for sequence identification and intra-species comparison. Phylogenetic tree was also constructed using tools present in the BLAST results page (Johnson *et al.,* 2008).

Submission of obtained sequence (query) to the NCBI

Figure 1: QR (Quick response) code can be scanned to see the obtained sequence submitted to GenBank along with all the other identifiers related to the sequence

Based on BLAST results, the earthworm specimen was identified as *Metaphire posthuma*and after identification the nucleotide sequence was submitted to GenBank and the accession number PP832872 was obtained. The nucleotide sequence can be reached online by scanning the following quick-response code presented as figure 1.

Phylogenetic tree construction

Following the Neighbor-Joining method, an unrooted phylogenetic tree was constructed using 13 sequences (including the COI sequence of *Metaphire posthuma* collected from Ranchi) to infer the evolutionary relationship (Saitou and Nei, 1987). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) is shown next to the branches (Felsenstein, 1985). The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al.,* 2004), and are in the units of number of base substitutions per site. This analysis involved 13 nucleotide sequences. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 660 positions in the final dataset. Evolutionary analyses were conducted in Molecular Evolutionary Genetics Analysis - MEGA X (version

10.2.6, build 10210527-x86_64 (Windows 11)(Kumar *et al.,* 2018).

Distance Matrix Calculation

The number of base substitutions per site from between sequences was calculated. Analyses were conducted using Maximum Composite likelihood model (Tamura *et al.,* 2004). The analysis involved 13 nucleotide sequences. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 660 positions in the final dataset. Evolutionary analyses were conducted in MEGA X (Kumar *et al.,* 2018).

RESULTS AND DISCUSSION

Morphology based features to identify *Metaphire posthuma* suggested by Chang *et al.,* (2017)

Size 60–210 mm by 3–8 mm.

Segment numbers 91–140.

Color of live specimen's brown.

Male pores paired in XVIII, in an invagination.

Post-clitellar genital markings paired on setal circle on XVII, XIX, slightly median to male pores.

Spermathecal pores four pairs, on the posterior margin of segments, just in front of 5/6/7/8/9.

Pre-clitellar genital markings absent. Female pore single in XIV. First dorsal pore 11/12 or 12/13.

Anatomical morphology based features for confirmation

Spermathecae four pairs in VI–IX, each with a short, stout duct and oval or heart-shaped ampulla; diverticulum stalk short and slender, seminal chambers usually longer than stalks.

Prostate glands paired, extending through some or all of XV–XXI; accessory glands in XVII and XIX, corresponding to external genital markings.

Intestinal caeca paired in XXVII, simple, extending anteriorly to XIV.

Remarks.

The first record of *M. posthuma* in India in 1909 by Michaelsen

***Pheretima posthuma* (Vaillant, 1868)**

1868. *Perichaeta posthuma*, L. Vaillant, *Ann. Sci. Nat.* (5), X, p. 228. (Type locality, Java, Types in the Paris Museum)

1883. *Megascolex affinis*, Beddard, *Ann. Mag. Nat. Rist.* (5), XII, p. 214.

1889. *Megascolex posthuma*, Vaillant, *Hist. Nat. Annel*. III, (1) p. 72.

1895. *Perichaeta posthuma*, Beddard, *ltlonog.* P. 424. 1900. *Pheretima posthuman*, Michaelsen, *Das Tierreich*, X, p. 295.

1900. *Amyntas posthuma*, Beddard, *Proc. Zool. Soc.* London, 1900, p. 641.

1901. *Amyntas posthumus*, Beddard, *Proc. Zool. Soc.* London, 1901, p. 196.

1902. *Pheretima posthuma*, Beddard and Fedarb, *Proc. Zool. Soc. London*, 1902, p. 164. (Coelomic pouches.)

1903. *Pheretima posthuma*, Michaelsen, *Geogr. Verbr*. p. 98.

1909. *Pheretima posthuma*, Michaelsen, *Mem. Ind. Mus.* I, pp. 110 and 189.

1910. *Pheretima posthuma*, Michaelsen, *Abh. Nat. Ver.* Hamburg, XIX (5), p.12.

1911. *Pheretima posthuma*, Lloyd and Powell, *J. Bombay Nat. Hist. Soc.* XXI, pp. 289 and 291.

1913. *Pheretima posthuma*, Stephenson, *Trans. Roy. Soc.* Edinburgh, XLIX, p.764. (Circulatory System.).

1914. *Pheretima posthuma*, Stephenson, *Bec. Ind. Mus.* X, pp. 323 and 342.

1915. *Pheretima posthuma*, Stephenson, *Mem. Ind. Mus.* VI, pp. 37 and 99.

1916. *Pheretima posthuma*, Stephenson, *Bee. Ind. Mus*. XII, p. 334.

1917. *Pheretima posthuma*, Stephenson, *Quart. J. Mic. Sc*i. LXII, p. 261. (Pharyngeal gland cells.).

1917. *Pheretima posthuma*, Stephenson, *Ree. Ind. Mus.* XIII, p. 385.

1918. *Pheretima posthuma*, Thapar, *Rec. Illil. Mus*. XV, pp. 71 and 74. (Lymph glands and coelomic organs.)

1919. *Pheretima posthuma,* Bahl, *Quart. J. Mic. Sci.* LXIV, pp. 76 and 109. (Nephridia and septa.).

1920. *Pheretima posthuma*, Stephenson, *Mem. Ind. Mus* VII, p. 222.

1921. *Pheretima posthuma,* Bahl, *Quart. J. Mic. Sci.* LXV, pp. 349 and 354. (Circulatory system.)

1922. *Pheretima posthuma*, Stephenson, *Bee. Ind. Mus* XXIV, p. 434.

1922. *Pheretima posthuma*, Bahl, *Quart. J. Mic. Sci*. I.JXVI, p. 56. (Cocoons.)

1923. *Pheretima posthuma*, Stephenson, Oligochaeta, in *F. B. 1. Series*, p. 309.

1924. *Pheretima posthuma*, Stephenson, *Bee. Ind. Mus*. XXVI, p. 340.

1924. *Pheretimap osthuma*, Stephenson, *Proc. Roy. Soc. London*, B, XCVII, p. 180. (Blood glands.)

1926. *Pheretima posthuma*, Stephenson, *Rec. Ind. Mus*. XXVIII, p. 258. 1.

1958. *Pheretima posthuma*, Gates 1958: 31; 1982: 61.

1972. *Pheretima posthuma* Gates, *Trans. Am. Phil. Soc.*, 62 (7): 2012; 1972.

1972. *Metaphire posthuma*, Sims and Easton 1972: 239.

2004. Reynolds and Wetzel, 2004: 88.

2008. Reynolds 2011: 281

Diagnosis. Length 60-190 mm, diameter 3-8 mm, 91-124 segments. Epilobic Prostomium with usually opened tongue. First dorsal pore in 12/13. Annular Clitellum on *xiv-xvi*. Setae 106-129 on *viii*, 63-75 on *xii*, 60-95 on *xx,* 36-44 between spermathecal pores, 16-22 between male pores. Presence of male pores on *xviii*, 0.25 body circumference apart. Single and median female pore presetal on *xiv*. Paired spermathecal pores, minute in 5/6, 6/7, 7/8 and 8/9. Paired genital markings,

usually on setal arcs of *xvii* and *xix* slightly median to male pore lines, sometimes on *xvi* and a few segments posterior to *xix*.

Muscular septa on 5/6-8/9, absent in 9/10. Intestine begins in *xv*; Paired intestinal caeca, simple, originating in *xxvii* and extending anteriorly to *xxiv*; simple and lamelliform typhlosole. *Xiii* segment bears last pair of hearts. Testes Holandric and male funnels enclosed in unpaired sacs, those of *x* ventral, those of xi vertically U-shaped; seminal vesicles in *xi* and *xii*, those of *xi* small, included in the testis sac; pseudovesicles small, in *xiii*. Paired spermathecae in *vi-ix,* each with an ental diverticulum of variable length, Genital marking glands sessile.

Distribution. India: Jharkhand, Punjab, Maharashtra, Bihar, Uttar Pradesh, Rajasthan, Orissa, West Bengal, Madhya Pradesh, Andaman and Nicobar Islands. Thailand, Burma, Bangladesh, Indonesia, Malaya Peninsula, S.E. Asia, Formosa,

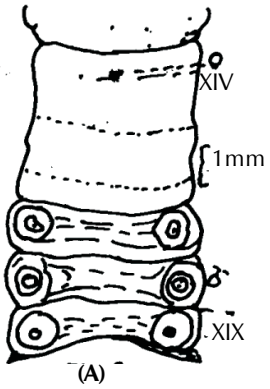

***Figure 1: Metaphire posthuma* Vaillant - Male Genital Region**

Figure 2: Metaphire posthuma Vaillant - Spermatheca

Philippines, and U S A.

Material examined. 15 clitellate worms.

Habitat. It was collected from sandy loam soil with a high organic content (>5%). The worm was present in subsoil at a depth of 10-20 cm. It is usually found in grassland, lawn and kitchen garden.

Economic importance: It is most commonly used as a laboratory material in India

Molecular sequence analysis of earthworm sample

DNA extraction, PCR amplification and sequencing resulted into a single discrete PCR amplicon of 661 bp of COI region using specific forward and reverse primer using aligner software.

The obtained sequence along with the accession number (GenBank) is listed below.

>PP832872

ggtacactatacttcatcttaggagtttgagccggaataattggagcaggaataa gacttcttattcgaattgaactaagacaaccaggatcctttctagggagagacca actatataacactattgttaccgcacatgcgtttttaataattttctttttagtaatgcc cgtatttattggtgggtttggaaattgattactacctcttatacttggaaccccaga catagcatttccacgactaaataacataagattctgattgctcccgcc atcactaattttactcgttaggtcagccgccgtagaaaagggagcag gtactggatgaacagtataccc cccactag caaga aatattgc acacgc cggaccg tcagtagatctggcaatttttcacttcacctagctggtgcatcttcaattttgggg gcca ttaacttcattacaacagtaattaatatacgatgatctggactacg attagaacgaatcc cacttttg tgtgagcagtcgtaattactgtagtactactattatta tcattaccg gt gtt agc tg gg gccatt ac catac ttct a ac agaccga aatcta aa cacttc att cttt gaccc agctg gg gg cg gg gatccaattc tataccaacacttattc

Previous studies (Kushwaha *et al*., 2015; Kim *et al*., 2017; Gabriella *et al*., 2019) have shown that the size of COI gene after PCR amplification, in case of different earthworm species ranges between 660 to 700 bp. The properly and successfully generated DNA barcode sequence of mitochondrial cytochrome C Oxidase – 1 (CO1) were subjected to BLAST as stated above, to search the most identical sequences deposited in the NCBI GenBank public databases.

The nucleotide 'query' (PP832872) was compared with the nucleotide database using BLASTn, with parameters selected as SearchSet: Standard Database; Program selection: Highly similar sequences (megablast) (Johnson *et al*., 2008; NCBI-Help, 2024).Megablast is used for sequence identification and intra-species identification.

A total of 12 nucleotide sequences of *Metaphire posthuma* from the GenBank matched with that of our COI sequence *Metaphire posthuma* (Table 1), where the total score ranged from 765 to 1219.Thehighest value of total score 1219 each was obtained with (top two) sequences of *Metaphire posthuma* of Bangkok (Thailand) and Tianjin (China),in both the cases the query cover percentage and percentage identity were 100%, suggesting that the *Metaphire posthuma*found in Ranchi (India) is closely related to the former earthworms (Johnson *et al.,* 2008).

The other 10 hits obtained in BLAST search had their origin in Mizoram (India), Hanoi (Viet Nam), and Bangkok (Thailand). The differences and similarity between our COI sequences with the COI sequences of the 12 sequences selected as per the BLAST hist can be easily seen in the NCBI MSA (Multi Sequence Alignment) chart presented as Figure 4.

The query cover and percentage identity between query and the 12 subject sequences has been graphically presented as Figure 5. Based on the high similarity or our *Metaphire posthuma* COI sequence with the 12 nucleotide sequences (COI) of *Metaphire posthuma* obtained from GenBank, our specimen collected from Ranchi (India) is confirmed as *Metaphire posthuma*(Celik *et al.,* 2024; Raghuram *et al.,*2024).

The COI sequences from the 13 strains of *Metaphire posthuma*

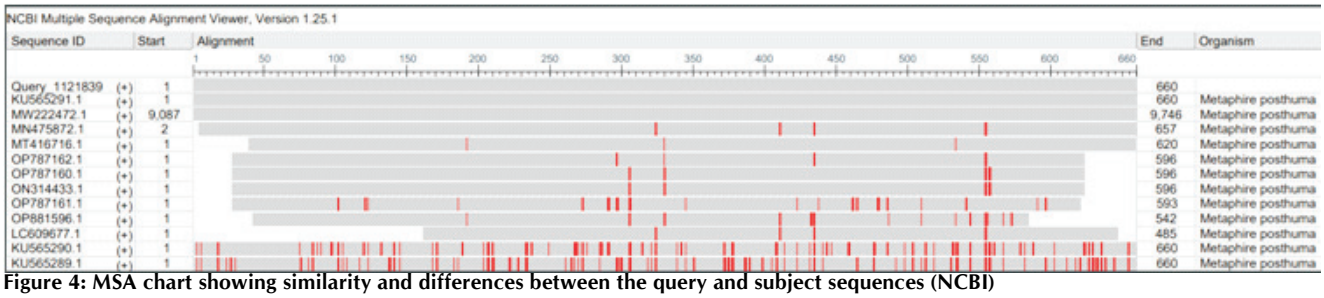

Figure 4: MSA chart showing similarity and differences between the query and subject sequences (NCBI)

Table 1: List of sequences taken from GenBank for construction of Phylogenetic tree with total score, query cover, E value and Percentage Identity obtained through BLASTn search; Country and Accession ID

Scientific Name	Total Score	Query Cover	E value	Per. Ident	Accession	Country
Metaphire posthuma	1219	100%	0	100.00%	KU565291.1	Thailand (Bangkok)
Metaphire posthuma	1219	100%	0	100.00%	MW222472.1	China (Tianjin)
Metaphire posthuma	1190	99%	0	99.39%	MN475872.1	India (Mizoram)
Metaphire posthuma	1129	93%	0	99.52%	MT416716.1	India (Mizoram)
Metaphire posthuma	1079	90%	0	99.33%	OP787162.1	Viet Nam (Hanoi)
Metaphire posthuma	1079	90%	0	99.33%	OP787160.1	Viet Nam (Hanoi)
Metaphire posthuma	1079	90%	0	99.33%	ON314433.1	Viet Nam (Hanoi)
Metaphire posthuma	990	89%	0	96.80%	OP787161.1	Viet Nam (Hanoi)
Metaphire posthuma	924	82%	0	97.42%	OP881596.1	India (Ludhiana)
Metaphire posthuma	874	73%	0	99.18%	LC609677.1	India (Raebareli)
Metaphire posthuma	815	100%	0	88.94%	KU565290.1	Thailand (Bangkok)
Metaphire posthuma	765	100%	0	87.58%	KU565289.1	Thailand (Bangkok)

Table 2: Estimates of evolutionary divergence between the sequences. The number of base substitutions per site from between sequences is shown. Analyses were conducted using Kimura 2-parameter model. This analysis involved 13 nucleotide sequences. Evolutionary analyses were conducted in MEGA X

Description of sequence		1	2	3	4	5	6	7	8	9	10	11	12	13
PP832872.1: Ranchi_(India)	1		0.00	0.00	3.63	3.15	1.96	1.96	1.96	1.93	3.02	4.01	0.12	0.14
KU565291.1: Bangkok_(Thailand)	2	0.00		0.00	3.63	3.15	1.96	1.96	1.96	1.93	3.02	4.01	0.12	0.14
MW222472.1: Tianjin_(China)	3	0.00	0.00		3.63	3.15	1.96	1.96	1.96	1.93	3.02	4.01	0.12	0.14
MN475872.1: Mizoram_(India)	4	3.63	3.63	3.63		3.58	4.74	4.64	4.64	4.71	5.46	5.29	3.50	3.72
MT416716.1: Mizoram_(India)	5	3.15	3.15	3.15	3.58		2.42	2.42	2.42	2.26	1.71	3.91	2.94	3.21
OP787162.1: Viet_Nam_(Hanoi)	6	1.96	1.96	1.96	4.74	2.42		0.01	0.01	0.04	2.51	4.45	1.89	1.90
OP787160.1: Viet_Nam_(Hanoi)	7	1.96	1.96	1.96	4.64	2.42	0.01		0.00	0.04	2.51	4.44	1.91	1.94
ON314433.1: Viet_Nam_(Hanoi)	8	1.96	1.96	1.96	4.64	2.42	0.01	0.00		0.04	2.51	4.44	1.91	1.94
OP787161.1: Viet_Nam_(Hanoi)	9	1.93	1.93	1.93	4.71	2.26	0.04	0.04	0.04		2.52	4.47	1.85	1.87
OP881596.1: Ludhiana_(India)	10	3.02	3.02	3.02	5.46	1.71	2.51	2.51	2.51	2.52		5.05	2.85	3.05
LC609677.1: Raibareli_(India)	11	4.01	4.01	4.01	5.29	3.91	4.45	4.44	4.44	4.47	5.05		4.48	3.92
KU565290.1: Bangkok_(Thailand)	12	0.12	0.12	0.12	3.50	2.94	1.89	1.91	1.91	1.85	2.85	4.48		0.11
KU565289.1: Bangkok_(Thailand)	13	0.14	0.14	0.14	3.72	3.21	1.90	1.94	1.94	1.87	3.05	3.92	0.11	

* Different colors denote the level of distance

listed in table 1 were used to prepare distance matrix using MEGA X software. Distance matrices are used in phylogeny as non-parametric distance methods. These distances are then reconciled to construct phylogenetic tree. Distance matrix methods of phylogenetic analysis explicitly rely on a measure of 'genetic distance' between the sequences being studied (Mount, 2004).

FASTA – aligned sequences of all the 12 accession ID were downloaded from the BLAST search results page. These sequences along with the sequence from the Ranchi strain were aligned using the MEGA X, and the phylogenetic tree was constructed using the neighbour-joining statistical method, bootstrap method was employed for the test of phylogeny (Sautou and Nei, 1987). The genetic distance in the resultant distance matrix (figure 6) ranged from 0.00 to 5.46.

Interestingly within India, the Ranchi strain is most distantly related to the Raibareli strain with a matrix score of 4.01.; but shows close relationships with 3 strains from Thailand, KU565291.1, KU565290.1 and KU565289.1, having matrix score of 0.00, 0.12 and 0.14 respectively. Even with the Tianjin (China) strain the Ranchi strain exhibit extremely close relation with a matrix score of 0.00.

It is seen that the Indian strains (Ranchi: PP832872.1; Mizoram:

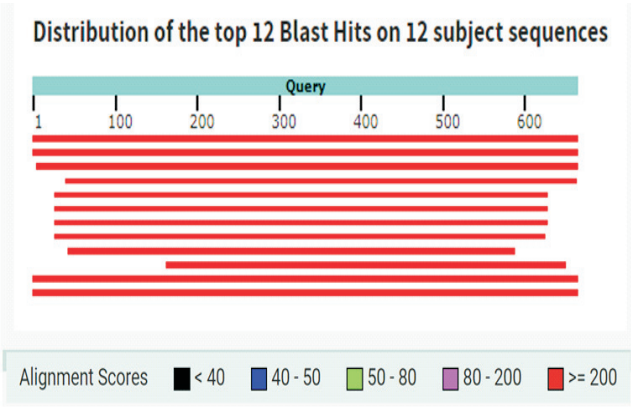

Figure 5: Graphic summary: Alignment scores of BLAST result

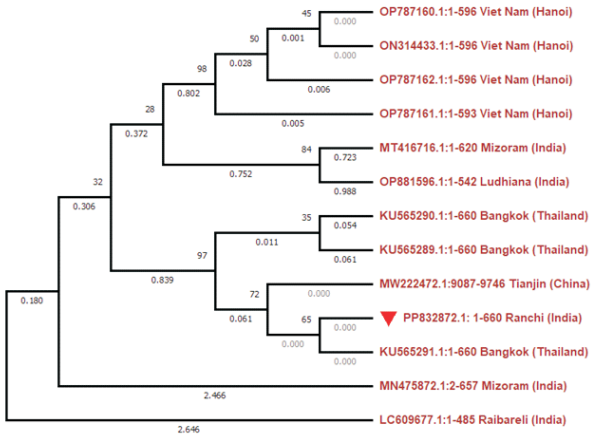

Figure 7: Phylogenetic tree constructed using the sequences obtained from GenBank listed in table 1

MN475872.1;Mizoram:MT416716.1;Ludhiana:OP881596.1; Raibareli: LC6090771) are distantly related to each other with a minimum matrix score of 1.71 and a maximum matrix score of 5.46 among them. This suggests that the Indian strains have wide genotypic variation and further study needs to be done related to intraspecific genotypic diversity.

Based on the study of morphological features and analysis of mitochondrial cytochrome oxidase subunit 1 (COI) nucleotide sequence, the earthworm sample collected from Ranchi (India) is confirmed as a member of Genus *Metaphire,* Species *posthuma*. Keen study of the phylogenetic tree and the matrix score obtained between Ranchi (PP832872.1)and the Tianjin (MW222472.1), and the Bangkok strains (KU565291.1; KU5652901.1 and KU565289.1), that all the 12 strains (Including the Ranchi strain) probably have Indian origin, since the Mizoram strain (MN475872.1) and Raibareli strain (LC609677.1) have a common ancestral origin, and all the rest 11 strains from Viet Nam (4), India (3), Thailand (3) and China (1) appears to have originated from the Mizoram (Indian) strain – MN475872.1).

The genetic variation in the specimen of the same species might be attributed to environmental impact and specifically to the habitat characteristics from where they have been collected. The study is helpful in analyzing cryptic diversity and also in ascertaining conclusive taxonomic level. Studies of Huang *et al*. (2007); King *et al*. (2008); Chang *et al*. (2009); Rougerie *et al*. (2009); Decaens *et al*. (2013), Khan *et al*. (2022), on barcoding of earthworm species have presented a similar conclusion for the specimen they studied.

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