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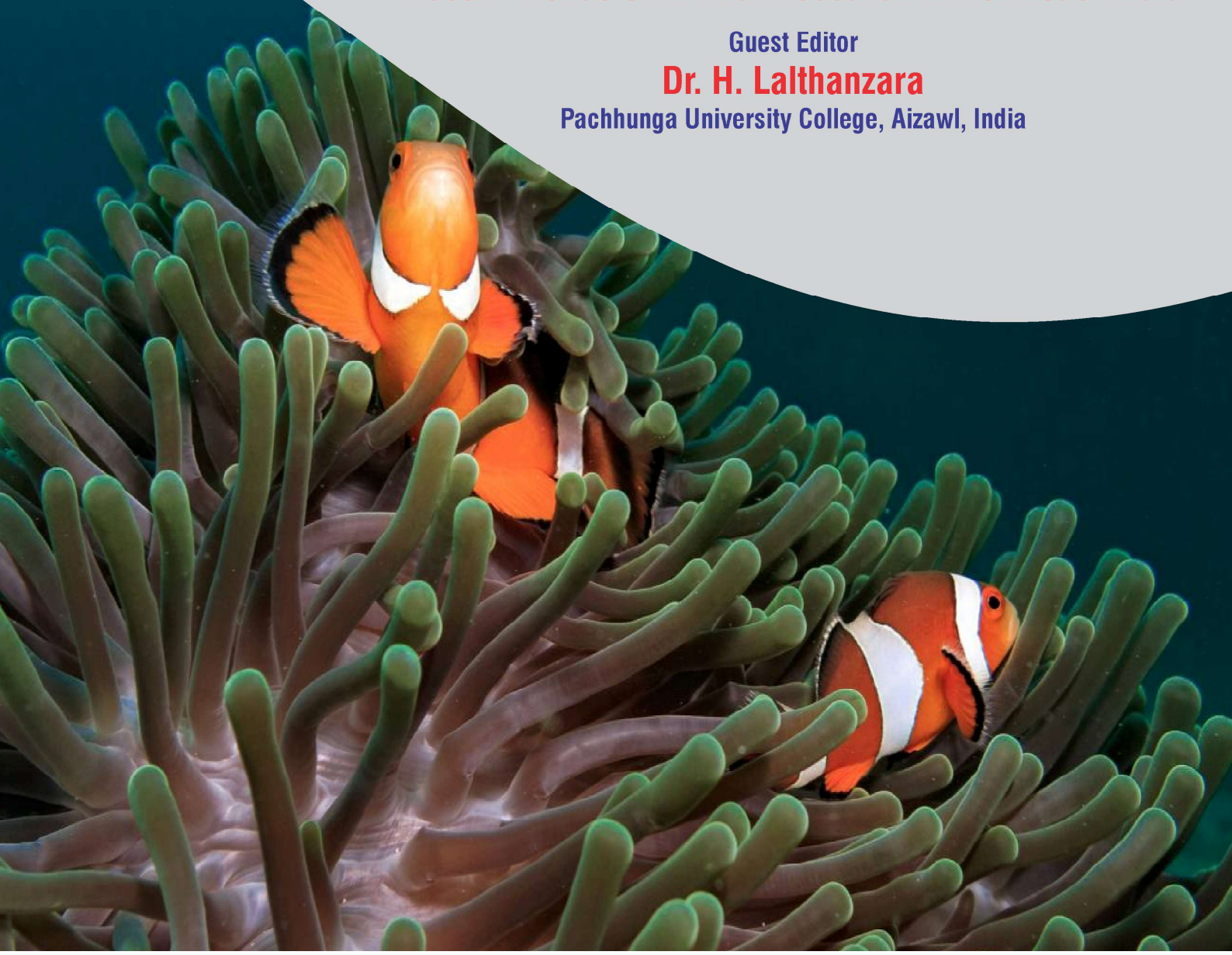

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Study on the *Amynthas* (Kinberg, 1867) earthworm (Megascolecidae: Oligochaeta) diversity through DNA barcoding from Northeast India

M. Vabeiryureilai[2], C. Zothansanga[1], M. Lalchhanhima[1], N.S. Kumar[2] and H. Lalthanzara[1]*

[1]Department of Zoology, Pachhunga University College, Aizawl-796 001, India
[2]Department of Biotechnology, Mizoram University, Aizawl-796 001, India

***Corresponding Author Email :** hzara.puc@gmail.com

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Abstract

Aim: To study species diversity of *Amynthas* (Kinberg, 1867) earthworm from Northeast India using mitochondrial CO1 gene by DNA sequencing.

Methodology: Earthworms were collected from different habitats of Northeast states of India. Morphological identification upto the genus was performed. Genomic DNA was extracted by CTAB method followed by PCR amplification of mtDNA CO1 gene. The amplicons obtained were sequenced by AB3500 Genetic Analyzers.

Earthworm sampling from eight Northeastern States of India. Specimens- fix & preserved in 100% ethanol and later 4% farmalin. Morpho-anatomical study for Genus *Amynthas* confirmation

↓

* Genomic DNA extraction from body tissue by CTAB method & PCR amplification using CO1 marker
* Sanger Sequencing by AB3500 Genetic Analyzers
*Sequence alignment and Blast Using ClustalW, MEGA-X, MrBayes Software

↓

10 sp. of *Amynthas* identified (>97% similarity) after BLAST in BOLD System & NCBI GenBank). Such as *Amynthas alexandri, A. corticis, A. diffringens, A. gracilis, A. hawayanus, A. hupeiensis, A. incongruus, A. morrisi, A. papulosus, & A. robustus.* Sequences submitted to NCBI GenBank.

Results: A total of 2355 *Amynthas* species were collected from 361 different sampling sites from various habitats of north east India. Sequencing of mtDNA CO1 marker followed by matching with BOLD system database confirmed the presence of ten different species of *Amynthas* earthworms such as *Amynthas alexandri, A. corticis, A.diffringens, A. gracilis, A. hawayanus, A. hupeiensis, A. incongruus, A. morrisi, A. papulosus* and *A. robustus.* The DNA sequence was submitted to NCBI GenBank. Among them, *A. corticis* was the most widely distributed species found in six states, followed by *A. diffringens* found in five states. *A. incongruus* was confined to Mizoram only and is a new record for the northeast region of India. Four species reported from Nagaland (*A. alexandri, A. diffringens, A. gracilis, A. morrisi*) are new records for the state, *A. diffringens* was new to Meghalaya, and *A. hawayanus, A. incongruus* and *A. populosus* were new records for the state of Mizoram. *A. hupeiensis* was a new record for Assam.

Interpretation: Based on the molecular analysis of mtDNA CO1 sequences, we have reported five newly recorded species for Nagaland, three for Mizoram and one each for Meghalaya and Assam states. Our results indicated the species richness of *Amynthas* earthworms. Many more species of *Amynthas* earthworms are yet to be contributed towards the species richness from Northeast India.

Key words: *Amynthas,* DNA sequencing, Earthworms, Genetic diversity, Northeast India

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Introduction

Earthworms are mostly terrestrial oligochaetes (Annelida: Clitellata) which contribute about 80% of total soil invertebrate biomass (Nainawat and Nagendra, 2001). From the globally estimated 6500 species of earthworms, only 3500 species have been described (Fragoso *et al.,* 1997). Dispersal of earthworms is usually uneven (Singh *et al.,* 2016) and their numbers varies according to type of soil (Curry, 1998) as well as ecological features, especially edaphic factors such as moisture and temperature (Kaleemurrahman and Ismael, 1981) and availability of organic matter in the soils (Namita and Swati, 2009). Recently, a metadata analysis has revealed that earthworm species diversity is higher in temperate region compared to tropical regions (Phillips *et al.,* 2019).

Amynthas (Kinberg, 1867) are group of earthworms classified under family megascolecidae (Oligochaeta: Annelida) comprise large number of species. The morphological features of earthworm species can be distinguished based on features like position and shape of clitellum, position of genital organs, number and arrangement of setae, position and number of spermathecal pores, dorsal pores, etc., (Stephenson, 1923; Gates, 1972; Sims and Easton, 1972; Julka, 1988). Morphologically, the genus *Amynthas* is characterized by annular clitellum in 14-16 segments, epilobous prostomium, three pairs of spermathecal pores in 5/6-7/8, first dorsal pore at 10/11 or at 11/12 in few specimens, single mid-ventral female pore at 14 segment, male pores are small circular pair at 18 segment, lateral to one or more pairs of post-setal small genital markings (small discs) (Stephenson, 1923; Gates, 1972). *Amynthas* earthworms can be recognized by a light-colored, smooth annular type of clitellum (Schult *et al.,* 2016). The BOLD system database has recorded 2,031 specimens and classified 185 different species of *Amynthas* (http://v3.boldsystems.org). China alone is known to harbor 302 *Amynthas* species (Sun, 2013). *Amynthas* earthworms are found in forests as well as local patchy soil with density >50 individuals per m2 (Gorres and Melnichuk, 2012). *Amynthas* are epi-endogeic worms that live underneath litter or in topsoil, whereby they alter the soil texture and eliminate organic materials from the soil surface (Archer, 2012; Gorres and Melnichuk, 2012; Greiner *et al.,* 2012; Ikeda *et al.,* 2015).

A significant number of new species are described every year. Due to proliferation of taxonomic descriptions of new species, distinctive morphological characteristics are not apparent at the species level and many cryptic species have been noted. For instance, recently a considerable number of re-description of earthworm species have been recorded for several taxa (Zhao *et al.,* 2017; Azama and Ishizuka, 2018; Hong *et al.,* 2018; Bozorgi *et al.,* 2019; Csuzdi *et al.,* 2019). Therefore, molecular intervention can serve as the only key to solve species validation (Blakemore, 2013). In India, 590 earthworm species and subspecies belonging to 67 genera have been recorded (Julka *et al.,* 2009). The extent of earthworm diversity in North-eastern India has also been reported by various researchers from different states such as Meghalaya (Mishra and Ramakrishnan, 1988; Halder, 1999; Kharkongor, 2018), Manipur (Haokip and Singh, 2012), Mizoram (Ramanujam *et al.,* 2004), Assam (Rajkhowa *et al.,* 2015), Tripura (Chaudhuri, 2012), Arunachal Pradesh (Julka, 1976; 1981; Halder, 2007), Nagaland (Thyug and Kakati, 2018) and a few diversity survey records from Sikkim (Soota and Halder, 1981, Subedi and Saxena, 2018; Subedi *et al.,* 2018a,b,c). However, no report on *Amynthas* species diversity based on the genetic diversity has been documented from this part of the Indo-Myanmar biodiversity hotspot region. Therefore, in lieu of the above, an attempt was made to characterize genetic diversity of *Amynthas* earthworms from north-east India.

Materials and Methods

The study area included all states of North-eastern India, *i.e.,* Arunachal Pradesh, Assam, Manipur, Meghalaya, Mizoram, Nagaland, Sikkim and Tripura as shown in Fig.1. The north-eastern region of India covers 2,62,379 km area (Rodgers and Panwar, 1988) is located between 22°N and 29°5'N latitude and 88°E and 97°30'E longitude and shares an international border with Bangladesh and Bhutan, China and Myanmar. In Northeast India, there are 28 National Parks of national as well as international importance. The region represents an important part of Indo-Myanmar biodiversity hotspot, one of the 25 global biodiversity hotspots (Chatterjee *et al*., 2006) and the eastern part of Himalayan Biodiversity hotspot.

The study was conducted between 20th May and 30th August, 2018. The earthworms were collected from the study sites by random method hand-sorting as well as opportunistic sampling was carried out from various sites, including house garden, forest, roadside, side drains, sewage disposal and grasslands of north eastern states (Fig. 1). Morphological identification of earthworms was performed in the Research and Instrumentation Centre, Department of Zoology, Pachhunga University College in consultation with earthworm taxonomic monographs (Stephenson, 1923; Gates, 1972; Julka, 1988) and studied under a stereo-zoom microscope (Optika SZN 8 with Optikam Pro 8 LT). The number of earthworms collected and the environmental variables were recorded and the specimens were preserved in 100% ethanol for further identification.

Specimens identified as *Amynthas* species were subjected to molecular characterization. Genomic DNA was extracted from the tissues of posterior part by using Cetyltrimethylammonium bromide method (Murray and Thompson, 1980). Molecular identification of earthworms was performed by using cytochrome c oxidase subunit (CO1) gene (forward primer LCO1490 5'- GGTCAACAAATCA TAAAGATATTGG - 3', reverse primer HCO2198 5'- TAGAATTAGAAGATCAACCAG - 3') (Folmer *et al.,* 1994). PCR reaction was prepared for 25 µl volumes which contained 0.8 µl (80 ng) DNA template, 12.5 µl PCR Master Mix (Takara Tlontekh), 11 µl nuclease-free water and 10 pmol (1 µl) of each - primer. PCR cycling consisted of an initial denaturation step at 94°C for 4 mins.

followed by 35 cycles for 1 min. at 94°C, 1 min. at 49°C and 1 min. at 72°C. And the last extension step was done at 72°C for 10 mins. The amplified products were checked in 1.5% agarose gel electrophoresis stained with Invitrogen SYBR Safe, with 100 bp DNA ladder as a marker. Sequencing was done by using 3500 Genetic Analyzer 8ch RUO, model no. 622-0100 (Thermo Fisher Scientific). DNA sequences were aligned, edited and analyzed using BLAST (Altschul *et al.,* 1990) and Bioedit sequence alignment editor (Hall, 1999). The DNA sequences were submitted to GenBank, NBCI.

Results and Discussion

In all, 2,355 earthworms were identified through morphological examination as *Amynthas* species (Megascolecidae). They were collected from 361 different sites of eight north-eastern states of India. The identified species were further confirmed by DNA sequencing. The analysis of generated DNA sequences revealed that 52 individuals belonged to 10 different species and the remaining specimens were further subjected to detail morpho-anatomical and molecular study. The nucleotide sequences of *Amynthas* were submitted to GenBank (NCBI) and their accession number were *A. alexandri* (MN151381), *A. corticis* (MT438695), *A. diffringens* (MT444902), *A. gracilis* (Mh191377), *A. hawayanus* (MT444905), *A. hupeiensis* (MT444908), *A. incongruus* (MT444907), *A. morrisi* (MT444903), *A. papulosus* (MT444906) and *A. robustus* (Mt444904) (Table 1). This study provides effective tool for identification of earthworm species based on mtDNA COI gene. For 52 *Amynthas* species identified, 99.9% of the COI identifications agreed with the morphological identifications by expert taxonomists. It was reported that over 98% of animal species show greater than 2% divergence and suggested that this was the threshold for spider identification (Hebert *et al.,* 2003). The estimated value of the shape parameter for the discrete Gamma Distribution is 0.2139. The substitution pattern

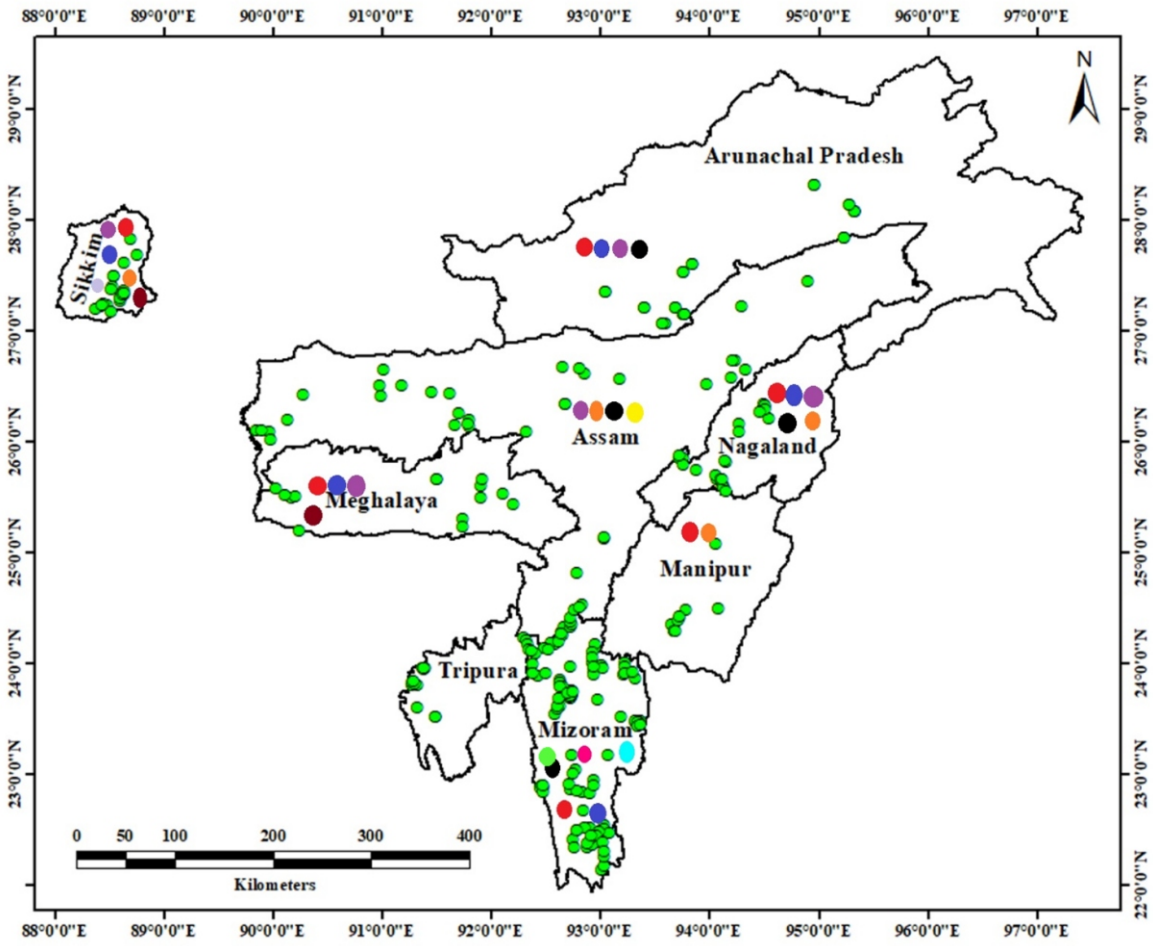

Fig. 1: Map of Northeast states showing collection sites along with their particular species found in each location indicating various colour codes: *A. corticis,* *A. gracilis,* *A. diffringens,* *A. morrisi,* *A. alexandri,* *A. robustus,* *A. hawayanus,* *A. papulosus,* *A. incongruus* and *A. hupeiensis.*

Table 1: Genetically identified *Amynthas* species of Northeast India

Amynthas species	AP	AS	MN	MG	MZ	NG	SK	TR	Accession No.
alexandri		+			+	+*		+	Mn151381
corticis	+		+	+	+	+	+		Mt438695
diffringens	+	+		+*		+*	+		Mt444902
gracilis	+			+	+	+*	+		Mh191377
hawayanus					+*		+		Mt444905
hupeiensis		+*							Mt444908
incongruus					+*				Mt444907
morrisi		+	+			+*	+		Mt444903
papulosus				+	+*				Mt444906
robustus				+			+		Mt444904

*New report from this location: AP (Arunachal Pradesh), AS (Assam), MN (Manipur), MG (Meghalaya), MZ (Mizoram), NG (Nagaland), SK (Sikkim), and TR (Tripura)

Table 2: Matrix of percentage pairwise nucleotide divergences with K2P distance on cytochrome c oxidase subunit I (COI) within *Amynthas* species

Species	NCBI Acc. No.	K2P-distance										
		1	2	3	4	5	6	7	8	9	10	11
*N. aibuhitensis*_1			0.030	0.027	0.027	0.026	0.030	0.026	0.029	0.026	0.023	0.027
*A. alexandri*_2	MT357030	0.338		0.021	0.021	0.021	0.022	0.020	0.022	0.020	0.021	0.021
*A. corticis*_3	MT438695	0.276	0.238		0.000	0.019	0.022	0.017	0.022	0.020	0.017	0.018
*A. diffringens*_4	MT444902	0.276	0.238	0.000		0.019	0.022	0.017	0.022	0.020	0.017	0.018
*A. gracilis*_5	MH191377	0.323	0.214	0.178	0.178		0.019	0.019	0.020	0.017	0.017	0.020
*A. hawayanus*_6	MT444905	0.337	0.215	0.212	0.212	0.199		0.021	0.018	0.020	0.020	0.018
*A. hupeiensis*_7	MT444908	0.263	0.195	0.147	0.147	0.181	0.190		0.022	0.018	0.016	0.017
*A. incongruus*_8	MT444907	0.311	0.226	0.209	0.209	0.200	0.171	0.200		0.021	0.021	0.020
*A. morrisi*_9	MT444903	0.302	0.212	0.191	0.191	0.165	0.208	0.191	0.215		0.016	0.019
*A. papulosus*_10	MT444906	0.266	0.192	0.149	0.149	0.165	0.182	0.145	0.181	0.148		0.018
*A. robustus*_11	MT444904	0.309	0.207	0.177	0.177	0.203	0.159	0.164	0.171	0.207	0.180	

The estimated value of the shape parameter for the discrete Gamma Distribution is 0.2139. Substitution pattern and rates were estimated under the Tamura (1992) model (+G) [1]. The nucleotide frequencies are A = 29.86%, T/U = 29.86%, C = 20.14%, and G = 20.14%. The maximum Log likelihood for this computation was -2678.989. This analysis involved 10 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. There was a total of 557 positions in the final dataset. Evolutionary analyses were conducted in MEGA X.

and rates were estimated under the Tamura (1992) model (+G). There were 557 positions in the final dataset coded with 209 amino acids. The sequences analysis revealed that 64% sequences were conserved regions as well as 36% variable sites with 24% parsimony information within the *Amynthas.*

The most variable sites among species were found to be 506-515 nucleotide regions, which signified different species for 10 variants (Fig. 2). The sequence analysis using DnaSP 6 software revealed that there were 10 haplotypes and haplotype diversity was 1.0 within the genus (Rozas *et al.,* 2017). Thus, the sequences amplified using mtDNA CO1 gene-specific primers identified 10 different species such as *A. corticis* (Kinberg, 1867), *A. diffringens* (Baird, 1869), *A. hupeiensis* (Michaelsen, 1895), *A. morrisi* (Beddard, 1892), *A. alexandri* (Beddard, 1900), *A. robustus* (Perrier, 1872), *A. hawayanus* (Rosa, 1891), *A. papulosus* (Rosa, 1896), and *A. incongruus* (Chen, 1933) (Table 1). The nucleotide frequencies are A = 29.86%, T/U = 29.86%, C = 20.14%, and G = 20.14%. The Kimura 2 parameter (K2P) genetic distance between *N. aibuhitensis* and other *Amynthas* were *A. alexandri* (33.8%), *A. corticis* (27.6%), *A. diffringens* (27.6%), *A. gracilis* (32.3%), *A. hawayanus* (33.7%), *A. hupeiensis* (26.3%), *A. incongruus* (31.1%), *A. morrisi* (30.3%), *A. papulosus* (26.6%) and *A. robustus* (30.9%). The K2P distance revealed that *Amythas* species were different from each other forming a monophyletic clade (Table 2, Fig. 3). Ten species of *Amynthas* showed that their genetic diversity located with their variable site at a particular nucleotide sequence (Fig. 3). *Amynthas* were isolated from an out-group (*Nereis aibuhitensis*) even though all these species belong to the same phylum. This result showed that the identified *Amynthas* in north-eastern India were different from each other, based on the genetically distinct populations from nucleotide sequences (Fig. 2). The levels of evolutionary divergence between sequences were obtained by a bootstrap procedure (1000 generates). The evolutionary divergence between *Amynthas* species ranged from 0.0018 to 0.25827, indicating that these species were different from each other (Table 2).

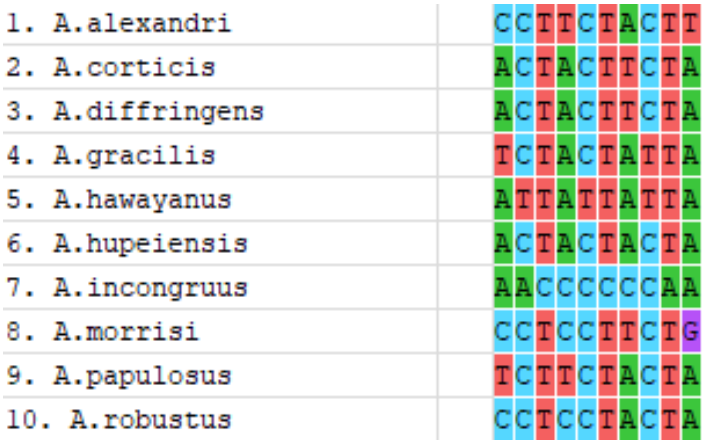

Fig. 2: Fragment of most variable site within 557 bp nucleotide sequences of mitochondrial CO1 gene, which signifies different species for a number of 10 variants identified within *Amynthas.*

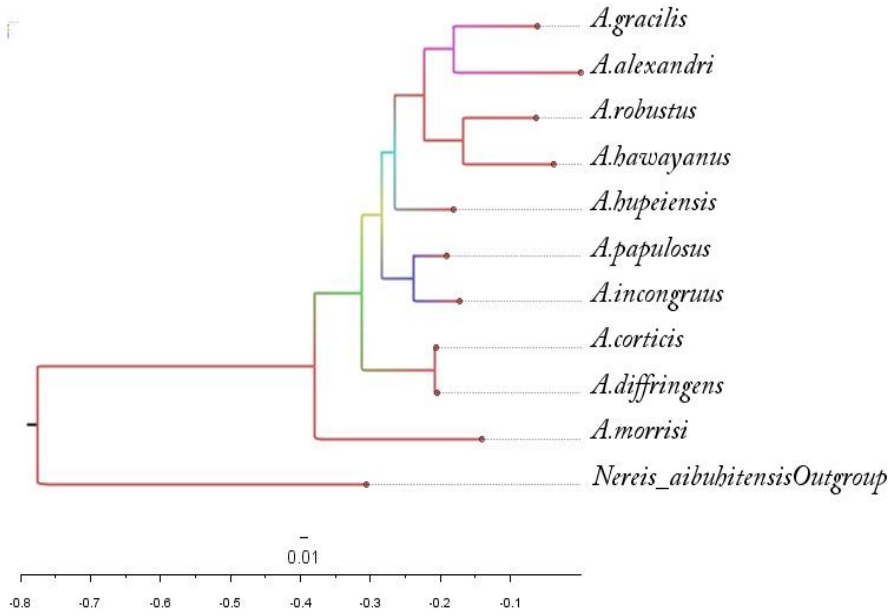

Fig. 3: Neighbour-joining phylogenetic tree based on K2P distances from 557 bp mtDNA COI gene sequences of *Amynthas* species. NJ tree was calculated in Mr Bayes based on the GTR+I+R with bootstrap values from 20000 replicates ($p<0.01$).

The distribution pattern of *Amynthas* species identified from this study revealed that state-wise, both Mizoram and Sikkim harboring six different known species had the highest species diversity, followed by Meghalaya and Nagaland with five species each (Fig.1). *A. corticis* was the most versatile species found in six states, followed by *A. diffringens* found in five states (Table 1). Halder *et al.* (2007) also recorded *A. diffringens* from five states of north-east India. Soota and Halder (1981) had reported the presence of *A. diffringens, A. hawayanus* and *A. morrisi* and Subedi *et al.* (2018b) reported *A. gracilis, A. robustus* and *A. corticis* from Sikkim. Kharkongor (2018) had recorded *A. cortices, A. gracilis, A. papulosus* and *A. robustus* from Meghalaya. Halder *et al.* (2007) also reported *A. morrisi* from Meghalaya. Ramanujam *et al.* (2004) had reported the presence of *A. alexandri* and *A. corticis* from Mizoram. Similarly, Haokip and Singh (2012) recorded two species (*A. cortices* and *A. morrisi*) in Manipur state. *A. diffringens* is the only *Amynthas* species recorded by Julka (1976) as *Pheretima diffringens* (its homotypic

synonym) from Arunachal Pradesh. Thyug and Kakati (2018) recorded *A. corticis* and one unidentified Amynthas species from Nagaland. Tripura harbored the lowest diversity with only one reported species (*A. alexandri*). Even though Tripura has a good number of earthworm species, however, out of 38 recorded earthworm species only *A. alexandri* has been reported from the genus Amynthas (Chaudhuri *et al.,* 2012). *A. robustus* has been reported from Meghalaya by Halder *et al.* (2007) and from Sikkim by Subedi *et al.* (2018c).

This study reported new records for some states of north-eastern India. Four species of Amynthas are new record from Nagaland, *viz., A. diffringens, A. gracilis, A. morrisi* and *A. alexandri*; one species from Meghalaya *i.e. A. diffringens*; four species from Mizoram *viz., A. hawayanus, A. incongruus, A. alexandri* and *A. papulosus.* In addition to the present record of ten species, many mtDNA CO1 sequences of *Amynthas earthworms* that we have documented were below 97% similarity when compared to BOLD systems and NCBI databases. It can also be inferred from these studies that more species would be identified from this site in future. Hence, this study affirms the unique and rich diversity of *Amynthas* species found in this biodiversity hotspot region.

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