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Preliminary Exploration of the Genetic Diversity and Structure of 107 *Amynthas morrisi* Specimens

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Abstract:

Earthworm plays an important role in soil ecological function, and more and more attentions were paid to the genetic variation of some representative earthworms, but not much for *Amynthas* species which were presumed mainly originated from China. In this study, based on the mitochondrial COI gene fragment, the genetic diversity, genetic structure, genetic differentiation and variation, and population history dynamics of 107 earthworm specimens of *Amynthas morrisi* (Bedard, 1892)) were analyzed. The results showed the 11 haplotypes in 107 samples, including five shared haplotypes and six unique haplotypes. The population had low genetic diversity (HD = 0.745 ± 0.027, π = 0.00800 ± 0.00216) and moderate genetic differentiation (F_{ST}= 0.21475, P < 0.001). Eastern hilly plain subregion (VIA) geographical population has the highest genetic diversity and the maximum genetic distance compared to other populations. 78.52% of the genetic variation was from within geographical populations and 21.48% from inter-populations. The divergence time estimation for 11 haplotypes divided into four lineages indicated that the genetic diversification of lineages occurred after Pliocene.

Keywords*: Earthworm, mitochondrial COI gene, genetic divergence, genetic structure, Amynthas morrisi*

I. INTRODUCTION

China is the country with the largest number of earthworm species in the genus *Amynthas*, which has been greatly developed in the south of China[1]. *Amynthas morrisi* (Bedard, 1892) belongs to Oligochaeta, Opisthopora, Megascolecidae. Its body length is 86-144 mm, width is 3.5-5.5 mm, segment number is 64-92, prostomium is 1/2 epilobous, dorsal pore start from 10/11, setae are thin, spermathecal pores are two pairs, located in 5/6/7, with small genital papillae present or absent in VII, and male pores paired in XVIII, with a pair of adjacent pre- and post-setal genital papillae medial to the male pore. *A. morrisi* is widely distributed in Southeast Asia (Myanmar, Thailand, Vietnam, Taiwan, Malaysia, Singapore, Sumatra, Hawaii), China, the United States, Mexico, South America, Caribbean, England, Spain, Italy, Pakistan, India, and Australia[2-4]. It is widely distributed in southern China in Jiangsu, Zhejiang (Lanxi, Tonglu, Fenshui, Linhai), Fujian (Xiamen), Taiwan (Taibei), Hong Kong, Hainan (Wanning), Chongqing (Nanchuan, Shapinba, Fuling, Jiangbei), Sichuan (Chengdu, Emei, and Leshan), Guizhou (Mt. Fanjing)[5].

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At present, there are lots of studies on the genetic and evolutionary relationship of the above orders of earthworm species[6-10]. Studies on genetic variation at population level mainly focused on the species of Lumbricidae in Europe and America[11-18]. However, there are few studies on the population genetics of the genus *Amynthas*, which plays an important role in the earthworm fauna of East Asia. We conducted a high density earthworm collection in South China, and try to uncover the possible lineages for those representatives Megascolecidae earthworms located in China[19, 20]. In this study, mitochondrial COI was amplified to analyze the genetic diversity and genetic structure of *A. morrisi* collected in southern China to further understand the historical population dynamics of this species.

In previous studies, we were accustomed to dividing geographical populations below earthworm species by the administrative boundaries of each province. However, the administrative boundaries did not have substantial significance for the geographical distribution of animals. Therefore, we attempted to refer to the demarcation standards from other animals in this study[21]. *A. morrisi* was divided into five geographic populations based on the above-mentioned demarcation criteria. We tried to study the genetic structure and relationships of these five geographic populations. In addition, we also respect the natural lineage based on molecular data, so we also studied the relationship between these potential lineages. We also want to see if there is a relationship between these lineages and geographic distribution.

II. MATERIALS AND METHODOLOGY

2.1 Sampling and Morphological Identification

Earthworms were collected in southern China in summer (Fig. 1). We finally collected 107 specimens of *A. morrisi*.

The fixation, preservation and morphological identification of specimens following the next steps: 1) the earthworm specimens were immersed in water and cleaned; 2) the cleaned earthworms were transferred to 10% alcohol solution until the earthworms were lazy, stop moving, and straight; 3) the anesthetized earthworms were transferred to 99.5% absolute ethyl alcohol; 4) the rigid ones were stored at 4 ℃.

The specimens were identified according to the morphological characteristics of *A. morrisi* described by Bedard (1892).

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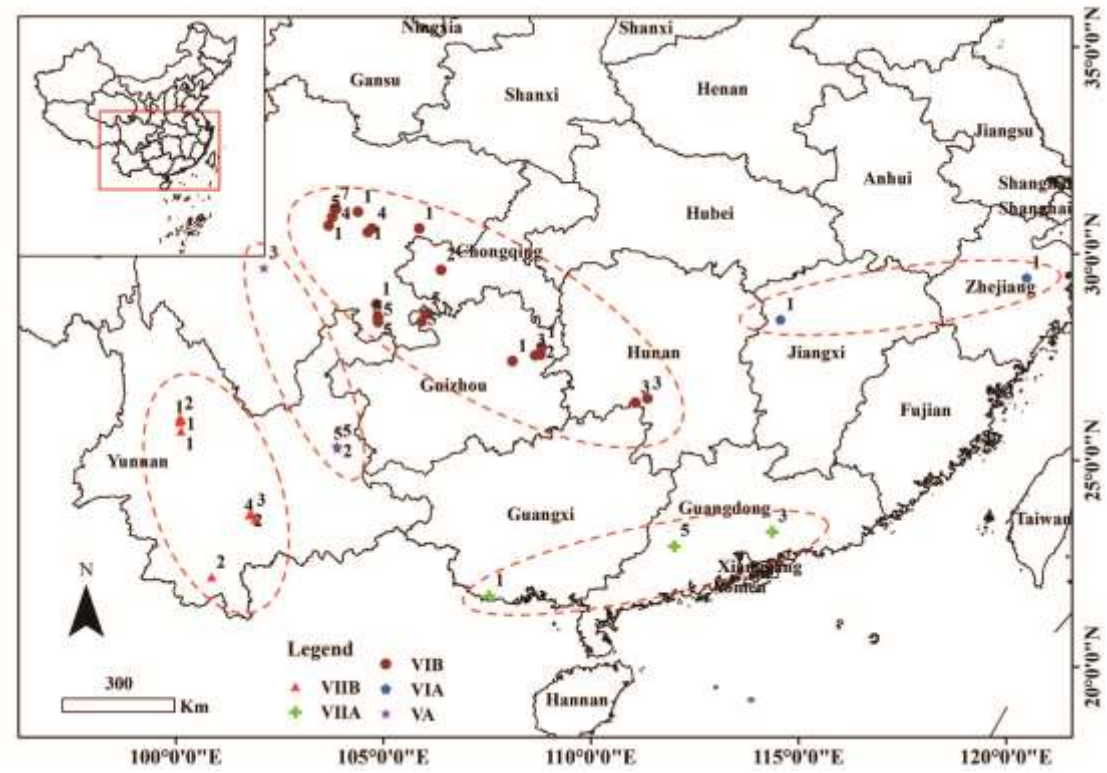

Fig. 1: The collection locations of specimens of A. morrisi

Note: the number in the box represents the number of specimens collected in this sample site, and the Roman number represents the zoogeographical affiliation in Zhang (2020)'s geographical divisions of animals (Zhang 2020).

2.2 DNA Extraction, Amplification and Sequencing

The extraction of total genomic DNA and the amplification of the mitochondrial COI gene were conducted as described in Yuan *et al.* (2020). Sequencing obtained in this research were deposited in GenBank (Supplementary Table 1).

2.3 Data Analysis

2.3.1 Phylogenetic analysis

Sequences obtained were aligned in the ClustalX2[22], and then were adjusted manually in BioEdit7.2.5[23]. The matched sequences were imported into jModelTest, and the likelihood values under different models were calculated[24]. The most suitable base substitution model (GTR + G) was selected by Akaike information criterion (AIC), and the NST value (NST = 6) needed for subsequent Bayesian tree setting was extracted. A Bayesian phylogenetic tree was constructed using Mrbayes3.2.6[25]. Using *Amynthas corticis* (Kinberg, 1867) as the outgroup, a random tree as starting tree, GTR + G as the model, Markov chain ran for 30000000 generations, sampling once per 1000 generations, removing the earliest obtained tree according to the proportion of 25%. The genetic distance was based on Kimura 2-parameter (K2P) distance model, bootstrap 1000 times repeated sampling test in MEGA7[26].

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2.3.2 Population genetic analysis

Haplotype diversity and nucleotide diversity were calculated in DnaSP5.10.1[27]. The number of haplotypes was calculated in DNAcollapser (FaBox 1.41). The haplotype network based on COI gene sequence was constructed in TCS1.21, and the connection limit was set to 50 steps[28]. Analysis of molecular variance (AMOVA) and neutral test were conducted in Arlequin3.1[29]. DnaSP5.10.1 was used to conduct the mismatch distribution analysis[27]. The species divergence time was estimated in BEAST1.8.4[30]. The generated file was opened in Tracer v.1.6, and the final tree was discarded in TreeAnnotator1.8.4.[30]. The divergence time and 95% confidence interval value of each node are annotated in the background tree.

III. RESULTS AND ANALYSIS

3.1 Species Identification

Earthworms were identified by morphology initially. The collected *A. morrisi* conformed to the morphological characteristics recorded by Beddard (1892), Chen (1931), Chen (1933), Chen (1936), Chen (1938), Gates (1939), Chen (1946), Tsai (1964), Gates (1972), Sims & Easton (1972), Chang et al. (2009), and Xu & Xiao (2011). The morphological identification result confirmed that all 107 specimens used in this study are *A. morrisi*.

After morphological identification, DNA barcoding data were blasted in GenBank. The results showed that the similarity between the samples used in this study and barcoding data in GenBank was more than 97%. Hence the 107 specimens used in this study are *A. morrisi* molecularly.

Genetic Diversity Analysis

According to the zoogeographical division of China, 107 *A. morrisi* specimens were filled into five geographic populations: Southwest mountain subregion (VA), Eastern hilly plain subregion (ⅥA), Western mountainous plateau subregion (ⅥB), Fujian Guangzhou coastal subregion (ⅦA) and southern Yunnan mountainous subregion (ⅦB)[21].

There were 537 bp conserved sites, 66 bp mutations and 63 bp polymorphic sites (accounting for 10.5% of the total length of the COI gene) in the mitochondrial COI gene segment which is 600 bp. There were no base deletion and insertion. The haplotype diversity (Hd) and nucleotide diversity (π) were 0.745 ± 0.027 and 0.00800 ± 0.00216. The highest Haplotype diversity (Hd) and nucleotide diversity (π) were in the Eastern hilly plain subregion (ⅥA), which were 1.000 ± 0.500 and 0.02000 ± 0.01000, respectively. The lowest haplotype diversity (Hd) and nucleotide diversity (π) were in the Southwest mountain subregion (VA), 0.248 ± 0.131 and 0.00165 ± 0.00087, respectively (Table 1). The results illustrated that the genetic diversity of the population in the eastern hilly plain subregion (ⅥA) was the highest, while that of the southwest mountain subregion (VA) was the lowest in these five geographical regions where we collected the specimens of *A. morrisi*.

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TABLE I: Haplotype and nucleotide diversity of different geographical populations in southern China

Population	Number of Haplotype (n)	Haplotypes (number of individuals)	Haplotype diversity (*Hd* ± SD)	Nucleotide diversity (π ± SD)
VA	2	Hap1(2),Hap6(13)	0.248 ±0.131	0.00165±0.00087
ⅥA	2	Hap1(1), Hap10(1)	1.000 ±0.500	0.02000±0.01000
ⅥB	8	Hap1(35), Hap2(7), **Hap4(3)**, **Hap5(3)**, Hap6(7), Hap9(7), Hap10(1), **Hap11(2)**	0.698 ±0.051	0.00910±0.00332
ⅦA	4	Hap1(1), Hap2(1), **Hap3(1)**, Hap6(3)	0.800 ±0.172	0.00511 ±0.00102
ⅦB	5	Hap2(1), Hap6(10), **Hap7(3)**, **Hap8(1)**, Hap9(1)	0.600 ±0.127	0.00200±0.00089
total	11	Hap1(39),Hap2(9),Hap3(1),Hap4(3),Hap5(3),Hap6(36),Hap7(3),	0.745±0.027	0.00800±0.00216
Total		Hap8(1),Hap9(8),Hap10(2),Hap11(2),		

The bold haplotype is the unique haplotype, and SD is the standard deviation.

3.2 Geographical Distribution of Haplotypes

The 107 specimens can be classified into 11 haplotypes (Table 1) and dispersed into five geographical regions. There were five shared haplotypes (Hap1, hap2, hap6, hap9, hap10). Hap1 was composed of 39 individuals widely distributed in 4 geographical populations, and hap6 was composed of 36 individuals widely distributed in 3 geographical populations. In addition, there are six exclusive haplotypes, among which Hap4, hap5 and hap11 are unique to ⅥB; Hap3 is unique to ⅦA; hap7 and hap8 are unique to the population of ⅦB in southern Yunnan.

The haplotype network map and phylogenetic NJ tree showed four lineages in the species, and had ever been reported in a conference report preliminary (Sun et al. 2020). Hap1 and hap6 are the two most important haplotypes.

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3.3 Genetic Distance of Intro- and Inter- Geographic Populations

The average genetic distance of different geographical populations in southern China was 0.0086, and the genetic distance between VIA population and other four geographical populations was the fast (0.012-0.014) (Table 2). The genetic differentiation index (FST) among different populations shown that there are very significant differences among VIB and VA, VIB and VIIB populations ($P < 0.01$), there are significant differences between VI a and VA, VII A and VA, VIIB and VIA, VIIB and VIIA populations ($P < 0.05$); there is no significant difference between VIIB and VA, VIB and VIA, VIIA and VIA, VIIA and VIB ($P = 0.171$-0.390).

TABLE II: The pairwise genetic differentiation FST value (lower triangle) and genetic distance (upper triangle) based on the Kimura 2-parameter model between the populations of *A. morrisi*

	VA	ⅥA	ⅥB	ⅦA	ⅦB
VA		0.012	0.008	0.004	0.002
ⅥA	0.66012*		0.014	0.012	0.013
ⅥB	0.22005* *	0.14145N		0.007	0.009
ⅦA	0.21223*	0.22029N	-0.03057N		0.005
ⅦB	-0.00367N	0.66316*	0.26734* *	0.28251*	

*:P<0.05; * *:P<0.01; N: no significance (P>0.05)

The results of AMOVA showed that 78.52% of the total variation was from intro-population and 21.48% from inter-population (Table 3). The results showed that the genetic variation of *A. morrisi* collected in this study in southern China mainly existed within the geographical population, and the level of genetic variation among populations was low.

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TABLE III: AMOVA analysis of *A. morrisi* in different geographical populations

	d.f	Sum of squares	Variance components	Percentage of variation
Among populations	4	41.915	0.56995 Va	21.48
Within populations	10 2	212.571	2.08403 Vb	78.52
Total	10 6	254.486	2.65398	

Fixation index, F_{ST}= 0.21475, P < 0.001

3.4 Population Expansion History

The nucleotide mismatch distribution analysis for the entire population showed a single clear peak, indicating the existence of population expansion. However, two neutral test parameters (Tajima's D and Fu's FS) were positive and negative, and the p-value did not reach a significant level (Fig. 2). Therefore, the results did not indicate whether or not the entire earthworm population had experienced an expansion in history.

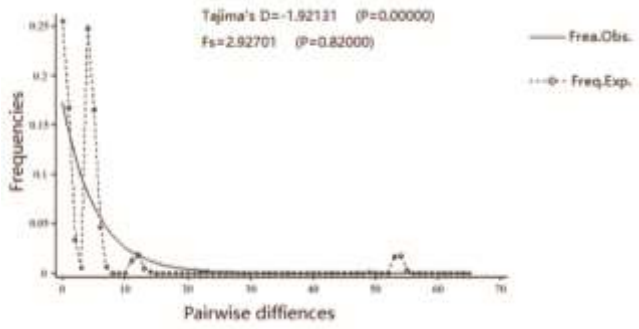

Fig. 2: Mismatch distribution of the entire population of 107 specimens of *A. morrisi* based on mitochondrial gene COI segment

3.5 Divergence Time Estimation

The structure of the phylogenetic tree we used here was consistent with that of the phylogenetic tree and haplotype network built previously[31], and it was divided into four branches. The divergence time estimation shows that the differentiation time of Linage D (node a) was 3.5605 Ma, that of Linage C (node b) was 0.8233 Ma, and that of Linage A and Linage B (node C) was 0.414 MA (Fig. 3).

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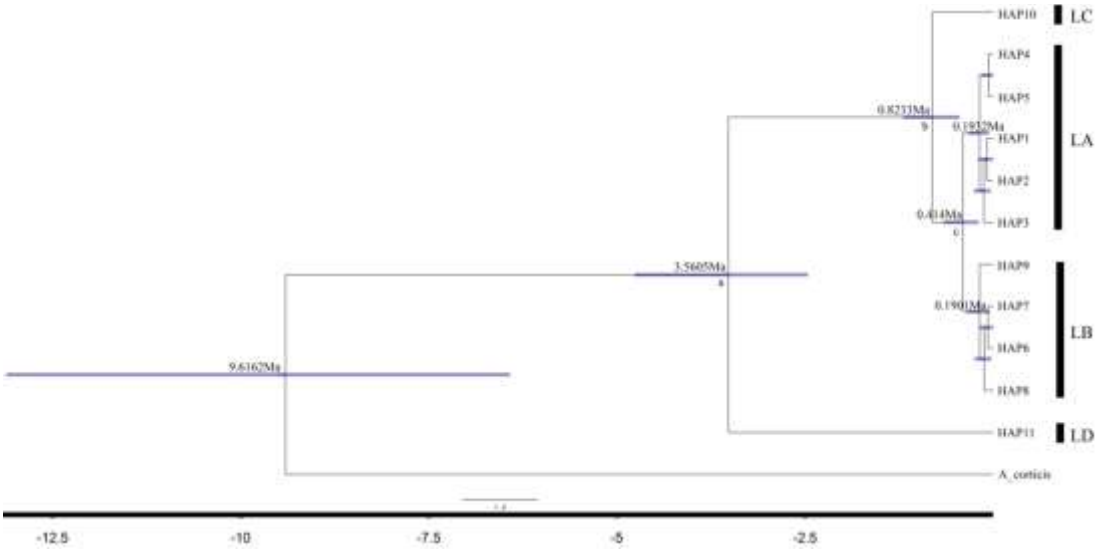

Fig. 3: Estimation of species divergence time of *A. morrisi* based on COI gene

IV. DISCUSSION

In COI data set of *A. morrisi*, 11% (66) of the 600 loci analysed were mutations, and 10.5% (63) were polymorphic sites. It is 45.5% (295bp) and 32.6% (211bp) mutations and polymorphic sites in *Hormogaster elisae* Álvarez, 1977[32], 12% mutations in *Aporrectodea icterica* (Savigny, 1826)[15], 34.2% polymorphic sites in *Rhinodrilus alatus* (Righi 1971)[13], 35.1% (231bp) polymorphic sites in *Eisenia nordenskioldi pallid* Malevič 1956[33], and 27.2% (276bp) polymorphic loci in *Lumbricus rubellus* Hoffmeister, 1843 based on the COI and ATP6 joint gene fragments[34]. Compared with other earthworm species, the genetic variation of *A. morrisi* was lower based on our collection in South China. It may be because of the lower gene flow of this special soil animal. Furthermore, as we know, *A. morrisi* is a cosmopolitan species[4], it is necessary to including all of the geographical populations all over the world in the further to estimate the genetic variation of this species.

The genetic diversity reflected by haplotype diversity and nucleotide diversity index showed that the genetic variation of *A. morrisi* was slightly less than that of other reported species. *H. elisae*, an endemic species in central Spain, varies from 0.67-0.92 in haplotype diversity (COI dataset) and 0.002-0.038 in nucleotide diversity in seven geographical populations in the central Iberian Peninsula[32]. *A. icterica*, collected from northern France, showed that the intra-population haplotype diversity (COI dataset) of seven geographical populations ranged from 0.135 to 0.742, and the nucleotide diversity ranged from 0.009 to 0.060[15]. *E. nordenskioldi pallid*, a widespread species in various climatic zones of Northern Asia, showed that 0.571 to 1.000 in haplotype diversity (COI dataset) and 0.1650 to 0.07556 in nucleotide diversity of 18 geographical populations[33]. In this study, the haplotype diversity (COI data set) of 5 populations of *A. morrisi* collected from southern China ranged from 0.248 to 1.000, and the nucleotide diversity ranged from 0.001 to 0.020. This could be the result of a low diffusion ability and gene flow of *A. morrisi*, or mixing effects caused by human activity.

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The geographic populations VIA has the highest genetic diversity. The *Amynthas* earthworm has the trend that originated from Southeast Asia or Indo-China Peninsula, then spread to China, then followed two routes, in which one branch goes to the southwest China, and another goes to the southeast China[10, 35]. So, it's easy to believe that the closer the population is from its origin, the more variation accumulates. However, the genetic diversity result of the geographic populations VIA shows a contrary conclusion, which recommends us two possibilities. The first possibility is *A. morrisi* has a different origination compared with most of *Amynthas* species. The second possibility is the "Zoogeographic map of China" is not suitable for defining the geographical population of earthworms.

When the K2P distance between samples based on the COI gene is less than 9%, it can be judged as the same species[36]. In this study, the K2P distance between samples or populations based on mitochondrial COI gene was 0.2% - 1.4%, which was less than the intra-specific difference criterion, indicating that all 107 specimens were the same species and the genetic difference was small.

The fixed index (Fst=0.00367) between populations were consistent with the average genetic K2P distance (0.002) , both show that the genetic distance between the adjacent subregion (VA and VIIB) is the closest. However, we have not proven whether it is homogenisation due to the large gene flows between the two geographically adjacent regions.

According to the present study and previous elementary report, Haplotype 1 and haplotype 6 are the most widely distributed haplotypes[31]. The original distribution area of these two haplotypes is covering five geographical regions: VA, VIA, VIB, VIIA, and VIIB. The phylogenetic relationship also shows that these two widely distributed haplotypes with the widest distribution were the last evolved lineages, perhaps because they are the most adaptable to their environment and can thrive in multiple regions. Due to the limited sample size, we failed to further explore the historical dynamics of each haplotype through mismatch distribution analysis and neutral test.

Most of the genetic variation of *A. morrisi* came from intro-population, but the genetic variation among populations was low. The molecular genetic variation of *H. elisae* mainly came from inter-population (92.59%), while the molecular variation within the population was only 7.41%[32]. Dong *et al.* (2020) reported that the genetic diversity of *Amynthas triastriatus*, and the diversity between and within lineages are 93.89% and 6.11%, respectively[19]. These results remind us that the "Zoogeographic map of China"[21], which is suitable for the geographical population division of large animals, is not suitable for defining the geographical population of earthworms, a soil animal.

The differentiation of *A. morrisi* and *A. corticis* occurred in the Miocene (9.6162 MA) of the late Tertiary, and the differentiation of the four lineages of *A. morrisi* occurred after the Miocene (3.5605 MA) of the Neogene, and the lineage LD was the first to differentiate in the Miocene (3.5605 MA), The differentiation of LC, LA and LB occurred in the Pleistocene of Quaternary (0.8233ma, 0.414ma). In the late Tertiary, the United continent disintegrated, Himalayan orogeny was frequent, mammals and angiosperms flourished. China was in the Western orogeny, the East was low and flat, lakes were widely

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distributed, and in the Quaternary, glaciers were widely distributed, and loess was formed. These geological structural events and biological evolution characteristics may be related to the intergenerational differentiation of *A. morrisi*[37]. In this study, the time of inter-specific lineage differentiation was earlier than that of Spanish species *H. elisae* (0.018 MA)[32]. However, the genetic divergence level of *A. morrisi* is lower than *H. elisae*. The earliest differentiation time (3.5605Ma) between lineages within *A. morrisi* population is earlier than that of another closely related species of the genus *Amynthas* (*A. triastriatus*, 2.97 Ma). Dong *et al.* (2020) considered that parthenogenesis could be an internal factor that influenced the differentiation and dispersal of *A. triastriatus*, and the Quaternary glaciation may have been one of the main factors that promoted the colonization of *A. triastriatus*[19]. In the present study, we did not give a precise analysis of the morphological features. However, certainly, we did not found the parthenogenesis morph in *A. morrisi*, and the two main lineages (LA and LB) differentiated in quaternary Pleistocene (0.414Ma), which reminds us the driving factor of lineage differentiation of these two species could be completely different. We will answer these questions in further research. *Amynthas*_YN2017 sp. is closely related to *A. morrisi*, Yang et al. (2020) revealed three lineages in *Amynthas*_YN2017 sp., and these three lineages coincided with the geographic distribution proximity of the sampling locations, which is different from *A. morrisi*[20]. Four detected lineages of *A. morrisi* are sympatric, and it is hard to find boundaries between lineages.

V. CONCLUSION

After morphological and DNA barcoding identification, 107 specimens were recognized in *A. morrisi*. The K2P distance between samples or populations based on mitochondrial COI gene was 0.2% - 1.4%. There were 537 bp conserved sites, 66 bp mutations and 63 bp polymorphic sites (accounting for 10.5% of the total length of the COI gene) in the mitochondrial COI gene segment which is 600 bp. The genetic diversity of the population in Ⅵ A was the highest in the five subregions. The genetic distance between VIA population and other four geographical populations (VA, Ⅵ B, Ⅶ A, Ⅶ B) was the farthest. The genetic distance between the adjacent subregion (VA and VIIB) is the closest. Most of the genetic variation of *A. morrisi* came from intro-population. However, the level of genetic variation among populations was low, which remind us that the "Zoogeographic map of China", which is suitable for the geographical population division of large animals, is not suitable for defining the geographical population of earthworms, a soil animal.

The 107 specimens can be classified into 11 haplotypes and four lineages. There were 5 shared haplotypes and six exclusive haplotypes. The differentiation of the four lineages of *A. morrisi* occurred after the Miocene (3.5605 MA) of the Neogene, and the lineage LD was the first to differentiate in the Miocene (3.5605 MA), the differentiation of LC, LA and LB occurred in the Pleistocene of Quaternary (0.8233ma, 0.414ma).

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REFERENCES

[1]. Chen, Y., Earthworms in China[M]. Beijing: Science Press: 1956.
[2]. Gates, G. E., Burmese earthworms: An introduction to the systematics and biology of megadrile oligochaetes with special reference to Southeast Asia. Transactions of the American Philosophical Society, 1972, 62 (7),pp 1-326.
[3]. Easton, E., Australian Pheretimoid Earthworms (Megascolecidae: Oligochaeta): a Synopsis With the Description of a New Genus and Five New Genera. Australian Journal of Zoology, 1982, 30 (4),pp 711-735.
[4]. Blakemore, R. J., Cosmopolitan Earthworms—A Global and Historical Perspective[M]. Hoboken: Wiley-Blackwell: 2009; p 257–283.
[5]. Xu, Q.,Xiao, N., Chinese terrestrial earthworm[M]. Beijing, China: China Agriculture Press: 2011.
[6]. Pop, A. A.,Cech, G.,Wink, M., etc., Application of 16S, 18S rDNA and COI sequences in the molecular systematics of the earthworm family Lumbricidae (Annelida, Oligochaeta). European Journal of Soil Biology, Nov, 2007, 43 pp S43-S52.
[7]. Buckley, T. R.,James, S.,Allwood, J., etc., Phylogenetic analysis of New Zealand earthworms (Oligochaeta: Megascolecidae) reveals ancient clades and cryptic taxonomic diversity. Molecular Phylogenetics and Evolution, Jan, 2011, 58 (1),pp 85-96.
[8]. Dominguez, J.,Aira, M.,Breinholt, J. W., etc., Underground evolution: New roots for the old tree of lumbricid earthworms. Molecular Phylogenetics and Evolution, Feb, 2015, 83 pp 7-19.
[9]. Csuzdi, C.,Chang, C.-H.,Pavlícek, T., etc., Molecular phylogeny and systematics of native North American lumbricid earthworms (Clitellata: Megadrili). Plos One, 2017, 12 (8),p e0181504.
[10]. Sun, J.,James, S. W.,Jiang, J., etc., Phylogenetic evaluation of *Amynthas* earthworms from South China reveals the initial ancestral state of spermathecae. Molecular Phylogenetics and Evolution, 2017, 115 pp 106-114.
[11]. Dupont, L., Perspectives on the application of molecular genetics to earthworm ecology. Pedobiologia, 2009, 2009, 52 (3),pp 191-205.
[12]. Knott, K. E.,Haimi, J., High mitochondrial DNA sequence diversity in the parthenogenetic earthworm *Dendrobaena octaedra*. Heredity, Oct, 2010, 105 (4),pp 341-347.

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[13]. Gailing, O.,Hickey, E.,Lilleskov, E., etc., Genetic comparisons between North American and European populations of *Lumbricus terrestris* L.. Biochemical Systematics and Ecology, Dec, 2012, 45 pp 23-30.
[14]. Siqueira, F. d. F.,de Cicco Sandes, S. H.,Drumond, M. A., etc., Genetic diversity and population genetic structure in giant earthworm *Rhinodrilus alatus* (Annelida: Clitellata: Glossoscolecidae). Pedobiologia, 2013, 2013, 56 (1),pp 15-21.
[15]. Torres-Leguizamon, M.,Mathieu, J.,Decaens, T., etc., Genetic Structure of Earthworm Populations at a Regional Scale: Inferences from Mitochondrial and Microsatellite Molecular Markers in *Aporrectodea icterica* (Savigny 1826). Plos One, Jul 8, 2014, 9 (7),p e101597.
[16]. Dupont, L.,Gresille, Y.,Richard, B., etc., Dispersal constraints and fine-scale spatial genetic structure in two earthworm species. Biological Journal of the Linnean Society, Feb, 2015, 114 (2),pp 335-347.
[17]. Ganin, G. N.,Atopkin, D. M., Molecular differentiation of epigeic and anceic forms of Drawida ghilarovi Gates, 1969 (Moniligastridae, Clitellata) in the Russian Far East: Sequence data of two mitochondrial genes. European Journal of Soil Biology, 2018, 86 pp 1-7.
[18]. Shekhovtsov, S. V.,Ershov, N. I.,Vasiliev, G., etc., Transcriptomic analysis confirms differences among nuclear genomes of cryptic earthworm lineages living in sympatry. Bmc Evolutionary Biology, 2019, 19 (S1),p 50.
[19]. Dong, Y.,Jiang, J.,Yuan, Z., etc., Population genetic structure reveals two lineages of *Amynthas triastriatus* (Oligochaeta: Megascolecidae) in China, with notes on a new subspecies of *Amynthas triastriatus*. International Journal of Environmental Research and Public Health, 2020, 17 (5),p 1538.
[20]. Yuan, Z.,Jiang, J.,Dong, Y., etc., Unearthing the genetic divergence and gene flow of the earthworm *Amynthas*_YN2017 sp.(Oligochaeta: Megascolecidae) populations based on restriction site-associated DNA sequencing. European Journal of Soil Biology, 2020, 99 p 103210.
[21]. Zhang, R., Chinese animal geography[M]. Science Press: 2020.
[22]. Thompson, J. D.,Gibson, T. J.,Frédéric, P., etc., The CLUSTAL_X Windows Interface: Flexible Strategies for Multiple Sequence Alignment Aided by Quality Analysis Tools. Nucleic Acids Research, 1997, 25 (24),pp 4876-4882.
[23]. Hall, T. A., BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/ NT. Nucleic Acids Symposium Series, 1999, 41 (41),pp 95-98.
[24]. Posada, D., ModelTest: Phylogenetic Model Averaging.. Molecular Biology and Evolution, 2008, 25 (7),pp 1253-1256.
[25]. Huelsenbeck, J. P.,Ronquist, F., Bayesian Analysis of Molecular Evolution Using MrBayes. Statistical Methods in Molecular Evolution, 2005, pp 183-226.
[26]. Kumar, S.,Stecher, G.,Tamura, K., MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets. Molecular Biology and Evolution, Jul, 2016, 33 (7),pp 1870-1874.
[27]. Librado, P.,Rozas, J., DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. Bioinformatics, 2009, 25 (11),pp 1451-1452.
[28]. Clement, M.,Posada, D.,Crandall, K. A., TCS: a computer program to estimate gene genealogies. Molecular Ecology, 2000, 9 (10),pp 1657-1659.
[29]. Excoffier, L.,Laval, G.,Schneider, S., Arlequin (version 3.0): An integrated software package for population genetics data analysis. Evolutionary Bioinformatics Online, 2007, 1 pp 47-50.
[30]. Drummond, A. J.,Suchard, M. A.,Xie, D., etc., Bayesian phylogenetics with BEAUti and the BEAST

Forest Chemicals Review
www.forestchemicalsreview.com
ISSN: 1520-0191
March-April 2022 Page No. 1223-1237
Article History: Received: 08 February 2022, Revised: 10 March 2022, Accepted: 02 April 2022, Publication: 30 April 2022

1.7. Molecular Biology and Evolution, 2012, 29 (8),pp 1969-1973.

[31]. Sun, J.,Jiang, J.,Yao, B., etc. Four genetic lineages were detected for earthworm *Amynthas morrisi* located in South China[A], In 2nd Internationa Conference on Frontiers of BiologicalI Sciences and Engineering (FSBE 2019)[C], 2020.

[32]. Novo, M.,Almodovar, A.,Diaz-Cosin, D. J., High genetic divergence of hormogastrid earthworms (Annelida, Oligochaeta) in the central Iberian Peninsula: evolutionary and demographic implications. Zoologica Scripta, Sep, 2009, 38 (5),pp 537-552.

[33]. Shekhovtsov, S. V.,Berman, D. I.,Bazarova, N. E., etc., Cryptic genetic lineages in *Eisenia nordenskioldi pallida* (Oligochaeta, Lumbricidae). European Journal of Soil Biology, 2016, 75 pp 151-156.

[34]. Giska, I.,Sechi, P.,Babik, W., Deeply divergent sympatric mitochondrial lineages of the earthworm *Lumbricus rubellus* are not reproductively isolated. BMC evolutionary biology, Oct 5, 2015, 15 (1),p 217.

[35]. Jiang, J. Taxonomy and molecular phylogeny of the family Megascolecidae earthworms from China[D]. Shanghai Jiao Tong University 2016.

[36]. Chang, C. H.,James, S., A critique of earthworm molecular phylogenetics. Pedobiologia, 2011, 54 (supp-S),pp S3-S9.

[37]. Jiang, J.,Qiu, J., Origin and evolution of earthworms belonging to the family Megascolecidae in China. Biodiversity Science, 2018, 26 pp 1074-1082.

Supplementary Table 1:

COI code	Morphological specimen code	Accession no.
Z100G_GZ	GZ201610-04C	MW479930
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Z10E_YN	YN201104-05B	MW479938
Z112M_HU	HU201614-05B	MW479888
Z113M_HU	HU201614-05C	MW479960
Z114B_SC	SCNQ2016-08	MW479906
Z117B_SC	SCCD2016-26	MW479961
Z118B_SC	SCCD2016-27A	MW479962
Z11E_YN	YN201104-05C	MW479965
Z124B_SC	SCPZ2015002-03B	MW479891
Z125B_SC	SCPZ2015002-03C	MW479885
Z131B_SC	SCPZ2015002-02B	MW479881
Z132B_SC	SCPZ2015002-02C	MW479953
Z133B_SC	SCPZ2015002-02E	MW479918
Z134B_SC	SCPZ2015002-02F	MW479901
Z135B_SC	SCPZ2015003-01B	MW479894
Z136B_SC	SCPZ2015003-01C	MW479879
Z137B_SC	SCPZ2015003-01D	MW479907
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Z13E_YN	YN201003-02	MW479955
Z140B_SC	SCPX2015001-01D	MW479882

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