Population Analysis Based on Mito-nuclear Sequences: Implication of *Dugesia japonica* Decline in Taihang Mountains, China

He-Cai Zhang, Tian-Ge Hu, Chang-Ying Shi, Guang-Wen Chen* and De-Zeng Liu

College of Life Sciences, Henan Normal University, Xinxiang 453000, China

ABSTRACT

Freshwater planarian *Dugesia japonica* is widespread in East Asia and has been used as a model animal in many research fields. Taihang Mountains is the demarcation line and indispensable treasure house of natural resources and ecological barrier in North China. In order to explore the genetic diversity, genetic structure and demographic history of *D. japonica* in Taihang Mountains, the mitochondrial *Cytb* and nuclear *ITS-1* sequences of 120 individuals from 20 populations were analyzed. The results showed that 46 haplotypes were detected in total, including 10 shared haplotypes and 36 private ones. The overall haplotype diversity (Hd) and nucleotide diversity (π) were 0.961 and 0.00157, respectively. The phylogenetic relationship and haplotype network analysis indicated that there was no obvious correspondence between the haplotypes and the geographical locations, revealing no obvious phylogeographic pattern. Analysis of molecular variance (AMOVA) suggested that the genetic differentiation of *D. japonica* in Taihang Mountains was very great (F_{ST}=0.405, *P*<0.01). This significant genetic variation mainly occurred within populations (59.52%), followed by 36.54% deriving from among populations. As for demographic analysis, positive Tajima's *D* and Fu's *Fs* values (0.250 and 1.657, respectively) were found in concatenated *Cytb/ITS-1* sequences as a whole for neutrality test. Moreover, the mismatch distribution was multimodal. Therefore, *D. japonica* in Taihang Mountains might have been undergoing a population decline, and conservation measures such as reducing human interference should be taken. We hope these findings will arouse conservation and management strategy regarding freshwater planarians and contribute to the biodiversity in the long run.

INTRODUCTION

Freshwater planarians which belong to phylum Platyhelminthes, class Turbellaria, order Tricladida are a group of bilaterian, dorsoventrally flattened, free-living invertebrate animals (Cebrià *et al.*, 2018; Zhang *et al.*, 2019a). Their striking regenerative ability has attracted the interest of scientists for centuries, and therefore led to their use as a classical model system of regeneration (Reddien and Alvarado, 2004). Meanwhile, due to their high susceptibility to xenobiotics, ease of acquisition and low maintenance cost, freshwater planarian has also been ideal test animal in toxicological research (Wu and Li, 2018). In addition, with the development of molecular marker and next generation sequencing technique, planarians with wide distribution and low dispersal capability has gradually become ideal model for exploring population genetics and phylogeography (Zhang *et al.*, 2019a). Investigations in this field will not only be helpful to clarify the genetic variation within a species and its interaction with the environment, thereby deepening the understanding of biological evolution process, but also provide theoretical basis for the conservation and development of species and contribute to formulate reasonable and efficient conservation measures (Avise, 2000). As a common species distributed in East Asia, *Dugesia japonica* is ubiquitous in most provinces of China and has large population size. At present, *D. japonica* as a model species has been mainly used to study regenerative biology and toxicology (Pagán, 2017; Reddien, 2018; Zhang *et al.*, 2018, 2019b; Ireland *et al.*, 2020). However, to our knowledge no work on the population genetics and phylogeography has been reported so far.

Taihang Mountains is located between the Loess Plateau and the North China Plain, which includes Beijing Municipality, Hebei, Shanxi and Henan provinces for an overall area of 136,500 km^2. It extends from the northeast to southwest with the elevation ranging from sea level to nearly 3000 m (Geng *et al.*, 2019). Due to the wide range of elevation and the typical earth-rocky features, Taihang Mountains possess highly heterogeneous features in

topography, soil, climate and vegetation (Zhao *et al.*, 2017; Fu *et al.*, 2018; Geng *et al.*, 2018). As the demarcation line of the second and third step of Chinese topography in north China, Taihang Mountains plays a vital role in ecological service, water conservation and climate regulation and is indispensable natural resources treasure house and ecological environment barrier (Hu *et al.*, 2019; Zhang *et al.*, 2019b).

Molecular markers based on the variation of DNA molecules can directly reflect genetic polymorphism at the DNA level (Sunnucks, 2000). Due to possessing the features such as strictly maternal inheritance, faster evolution rate and lack of genetic recombination, mitochondrial DNA has been widely used for studying phylogeography and population genetics (Pakendorf and Stoneking, 2005). The commonly used mitochondrial cytochrome b gene (*Cytb*) is an appropriate marker for exploring the genetic diversity and genetic differentiation of populations (Cantatore *et al.*, 1994). Nuclear DNA encompasses a large amount of genes and can theoretically meet the needs of phylogeographic research. Among them, the ribosomal internal transcribed spacer region (*ITS*) usually shows higher polymorphism and percentage of informative sites in studying intraspecies and closer interspecies relationships. At present, studies on population genetics and phylogeography of planarians using molecular markers *Cytb* and/or *ITS* have already been reported (Álvarez-presas *et al.*, 2011; Lazaro *et al.*, 2011; Solà *et al.*, 2013; Riesgo *et al.*, 2017).

In view of the unique geographical position and special topography of Taihang Mountains, we hypothesize that *D. japonica* in Taihang Mountains should have great genetic differentiation and show a certain geographical pattern. In addition, with the development of tourism in Taihang mountain area, we wonder to know whether the human activity would affect the population structure and population dynamics of *D. japonica*. To this end, the genetic diversity, genetic structure, and demographic history of *D. japonica* in Taihang Mountains based on mitochondrial *Cytb* and nuclear *ITS-1* were preliminarily analyzed in this paper.

MATERIALS AND METHODS

Sample collection

Planarians were collected in rivers, streams or springs across Taihang mountainous area from 2018 to 2019. For each population some samples were fixed and preserved in absolute ethanol for molecular analysis, while some sexually mature individuals were killed with 1% nitric acid and then fixed in Bouin's fluid for morphological diagnosis (because species identification for planarians is mostly based on the anatomical characters of genital organs). If there were no sexually mature adults in a certain population, juveniles were brought indoors and reared to adulthood to guarantee reliable species identification. Only the samples identified as *D. japonica* were used in this study. Figure 1 and Table I show the geographical distribution and the detailed collection information of

Table I. Collection information of *Dugesia japonica* populations used in this study.

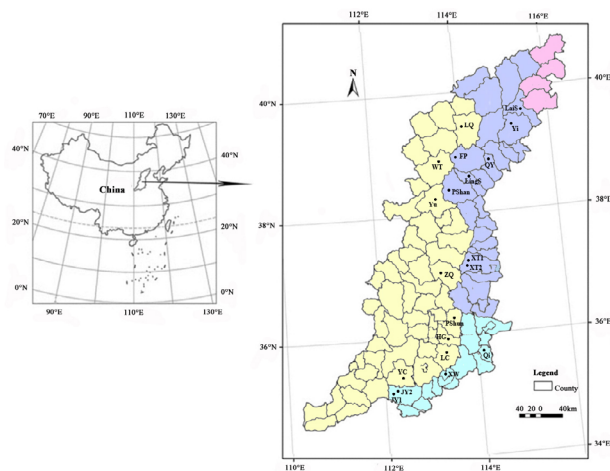| Population code	Location	Longitude and latitude	Altitude (m)	Collection date
WT	Wutai County, Shanxi Province	38°54' N/113°38' E	1391	2019-VIII
LQ	Lingqiu County, Shanxi Province	39°20' N/114°18' E	840	2019-VIII
Yu	Yu County, Shanxi Province	38°12' N/113°19' E	980	2019-VIII
ZQ	Zuoquan County, Shanxi Province	37°00' N/113°29' E	970	2019-VIII
PShun	Pingshun County, Shanxi Province	36°12' N/113°36' E	813	2019-IX
HG	Huguan County, Shanxi Province	35°55' N/113°35' E	250	2019-IX
LC	Lingchuan County, Shanxi Province	35°32' N/113°26' E	622	2019-IX
YC	Yangcheng County, Shanxi Province	35°14' N/112°26' E	575	2019-IX
Qi	Qi County, Henan Province	35°43' N/114°06' E	206	2018-IX
XW	Xiuwu County, Henan Province	35°28' N/113°20' E	490	2019-II
JY-1	Jiyuan County, Henan Province	35°15' N/112°11' E	791	2019-VII
JY-2	Jiyuan County, Henan Province	35°25' N/112°12' E	532	2019-VII
XT-1	Xingtai County, Hebei Province	37°20' N/113°54' E	1055	2018-X
XT-2	Xingtai County, Hebei Province	37°05' N/113°47' E	774	2018-X
Yi	Yi county, Hebei Province	39°23' N /115°15' E	433	2018-IX
QY	Quyang County, Hebei Province	38°56' N/114°33' E	371	2018-IX
LaiS	Laishui County, Hebei Province	39°39' N /115°23' E	430	2018-IX
LingS	Lingshou County, Hebei Province	38°41' N/113°51' E	835	2018-X
PShan	Pingshan County, Hebei Province	38°15' N/113°43' E	720	2018-X
FP	Fuping County, Hebei Province	38°55' N /113°48' E	978	2019-VIII

Fig. 1. Distribution map of *D. japonica* populations studied in Taihang Mountains. The red, blue, yellow and green areas represent Beijing Municipality, Hebei, Shanxi and Henan provinces, respectively.

D. japonica samples used in the current study, respectively. Voucher specimens were preserved in absolute ethanol and deposited in the Biological Museum of Henan Normal University (Xinxiang, China).

DNA extraction, PCR and sequencing

Total DNA was extracted from single planarian preserved in absolute ethanol by traditional phenol-chloroform method (Sambrook and Russel, 2016) or the DNeasy Blood and Tissue Kit (Qiagen, Germany) following the manufacturer's protocol. After quality checking and concentration measuring, the obtained DNA was stored at 4°C for immediate use or -2°C for later use.

A 317 bp fragment of *Cytb* was amplified by polymerase chain reaction (PCR) using the primer pair CytbS (5'-ATGTCTCTTTGAGGAGCTACTGT-3') and CytbA (5'-CTAAAAAATACCACTCAGGCTTTAT-3') designed by ourselves. As for the nuclear gene *ITS-1*, a fragment of 669 bp was amplified using the published primers ITS9F (5'-GTAGGTGAACCTGCGGAAGG-3') and ITSR (5'-TGCGTTCAAATTGTCAATGATC-3') (Álvarez-presas *et al.*, 2011). The PCR amplification conditions for *Cytb* were as follows: A total volume of 30 μL containing 15 μL TaqMix (dNTPs and Taq DNA polymerase plus, Vazyme Biotech, Nanjing, China), 12 μL ddH$_2$O, 1μL 10 μM forward and reverse primers (synthesized by Sangon Biotech, Shanghai, China) and 1μL DNA template reacted in a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, USA). The thermocycling procedure consisted of pre-denaturation at 94°C for 5 min, 34 cycles of denaturation at 94°C for 40 s, annealing at 45°C for 30 s and extension at 72°C for 1

min, followed by a final extension of 10 min at 72°C. As for *ITS-1*, the annealing temperature was 45°C, and other parameters were the same as *Cytb*. PCR products were confirmed as the target band by electrophoresis and then sent to Sangon Biotech (Shanghai, China) for sequencing with the corresponding amplifying primers.

DNA sequence analysis

Cytb and *ITS-1* chromatograms including sense and antisense were checked manually and assembled using Seqman in DNASTAR Lasergene v7.1 to obtain single consensus sequence. For the protein-encoding gene *Cytb*, the sequence accuracy was confirmed by translating into the amino acids via the online software EMBOSS Transeq (https://www.ebi.ac.uk/Tools/st/emboss_transeq/). MEGA 7 (Tamura *et al.*, 2011) was used to perform multiple sequence alignment. In order to avoid missing data, all sequences of *Cytb* and *ITS-1* were truncated to the same length of 317 bp and 669 bp, respectively, and the trimmed sequences of two genes were then manually concatenated into one data matrix (*Cytb/ITS-1*) for further analyses. All sequences of *Cytb* and *ITS-1* were submitted to GenBank and got the accession numbers.

In order to analyze the genetic diversity, number of haplotypes (N), haplotype diversity (Hd) and nucleotide diversity (π) were computed with the program DnaSP 5.0 (Librado and Rozas, 2009). Demographic history was explored by two different approaches in DnaSP 5.0. Firstly, Tajima's *D* and Fu's *Fs* statistics were used to test for neutrality. Then, the mismatch distribution to understand the demographic history was plotted. Using Arlequin 3.0 (Excoffier *et al.*, 2005), an analysis of molecular variation (AMOVA) was conducted for 20 geographical populations which were divided into two groups (Sourthern and Northern), and pairwise F_{ST} values to assess the genetic differentiation among populations were also quantified.

Phylogenetic topologies were reconstructed using Bayesian inference (BI) and maximum likelihood (ML) methods. The best-fit nucleotide substitution model TVM+I+G for the concatenated dataset was selected under Akaike Information Criterion (AIC) in the program jModeltest v2.1.7 (Darriba *et al.*, 2012). Bayesian analysis was performed via MrBayes v.3.2.1. (Ronquist *et al.*, 2012) based on the optimal model. Four Markov Chains (three heated and one cold) were run for ten million Markov chain Monte Carlo generations, sampling the chains every 500 generations. To verify the results, two independent runs were conducted. The initial 10% trees were discarded as burn-in samples. Remaining trees were used to generate a majority-rule consensus tree and Bayesian posterior probabilities (PP) (Huelsenbeck *et al.*, 2001). PhyML 3.0 (Guindon and Gascuel, 2003) was used to perform ML analyses. We used the pruning and

regrafting option to estimate better ML tree topology, and the non-parametric bootstrap with 1000 replicates to estimate the support for each internal branch of the ML phylogeny. Meanwhile, a haplotype network diagram was constructed using median-joining algorithm implemented in the Network 10.0 (Posada and Crandall, 2001).

RESULTS

Population genetic diversity

In the current study, 120 mitochondrial *Cytb* (GenBank accession numbers: MT767448-MT767567) and 120 nuclear *ITS-1* (GenBank accession numbers: MT767568-MT767687) sequences were obtained in *D. japonica* from 20 populations across Taihang Mountains, China. After alignment, trimming and concatenation, the final length of *Cytb/ITS-1* used for analyses was 986 bp. Altogether, 46 haplotypes were defined including 10 shared haplotypes (H1, H2, H5, H6, H8, H9, H11, H16, H29, H30) and 36 private ones (H3, H4, H7, H10, H12-H15, H17-H28, H31-H46). Among all the haplotypes, H30 was the most widely distributed and existed in 5 populations of JY1, WT, Yu, ZQ and YC. The overall haplotype diversity (Hd) and the nucleotide diversity (π) of 120 sequences were

0.961 and 0.00157, respectively. Except for population Yu (Hd=0.000), the Hd of other 19 populations were higher, ranging from 0.476 to 1.000. The π values were between 0 and 0.00195, with Yu showing the lowest value while XT-2 the highest (Table II).

Population genetic structure

The combined *Cytb/ITS-1* sequences of 20 *D. japonica* populations sampling across Taihang Mountains were subjected to AMOVA analysis. The results suggested that the genetic differentiation index (F_{ST}) of *D. japonica* in Taihang Mountains was 0.405 with the statistical difference being extremely significant ($P<0.01$). The major molecular variation (59.52%) occurred within populations, followed by 36.54% deriving from among populations; whereas variation among groups accounted for only 3.94% (Table III). The pairwise F_{ST} values computed from *Cytb/ITS-1* data were within the scope of -0.293 to 0.925, and the statistical differentiation reached significance ($P<0.05$) in 121 population pairs out of the total 190. The highest genetic variation was found between population HG and PShan (F_{ST}=0.925, $P<0.05$), followed by that between HG and Yu (F_{ST}=0.859, $P<0.05$) (Table IV).

Table II. Genetic diversity and neutral tests of *Dugesia japonica* based on *Cytb/ITS-1*.

Population code	Sample size (N)	Number of Haplotype (h)	Haplotype (Number of individuals)	Haplotype diversity (Hd)	Nucleotide diversity (π)	Tajima's D	Fu's Fs
LaiS	5	3	H1(1), H5(3), H6(1)	0.700	0.00093	-1.214	-1.261
Yi	4	3	H2(2), H3(1), H4(1)	0.833	0.00130	-0.744	-0.593
QY	7	2	H1(3), H2(4)	0.714	0.00147	2.351**	2.052**
FP	4	2	H2(1), H11(3)	0.833	0.00102	-0.858	-0.923
LingS	12	5	H2(1), H5(3), H9(1), H10(1), H11(6)	0.803	0.00088	-0.920	-1.110
PShan	5	2	H12(2), H13(3)	0.700	0.00038	1.840*	1.995**
XT1	4	4	H14(1), H15(1), H16(1), H17(1)	1.000	0.00185	0.598	0.968
XT2	4	4	H9(1), H18(1), H19(1), H20(1)	1.000	0.00195	0.050	0.462
Qi	7	4	H5(4), H6(1), H7(1), H8(1)	0.714	0.00061	-1.748**	-2.032**
XW	9	9	H21(1), H22(1), H23(1), H24(1), H25(1), H26(1), H27(1), H28(1), H29(1)	1.000	0.00162	0.864	1.001
JY1	4	4	H30(1), H31(1), H32(1), H33(1)	1.000	0.00184	0.578	0.927
JY2	4	3	H16(2), H34(1), H35(1)	0.833	0.00103	-0.874	-0.926
LQ	8	3	H5(2), H6(5), H11(1)	0.607	0.00127	0.675	0.686
WT	5	2	H30(4), H36(1)	0.700	0.00146	-1.273*	-1.388**
Yu	4	1	H30(4)	0	0	0.000	0.000
ZQ	12	4	H8(1), H30(2), H37(2), H38(7)	0.652	0.00037	-2.250**	-3.050**
PShun	5	5	H16(1), H39(1), H40(1), H41(1), H42(1)	1.000	0.00032	-0.868	-0.905
HG	5	2	H29(2), H43(3)	0.600	0.00001	1.225	1.157
LC	5	3	H6(3), H45(1), H46(1)	0.700	0.00148	-1.273*	-1.388**
YC	7	2	H30(2), H44(5)	0.476	0.00048	0.973	1.697*
Total	120	46		0.961	0.00157	0.250	1.657

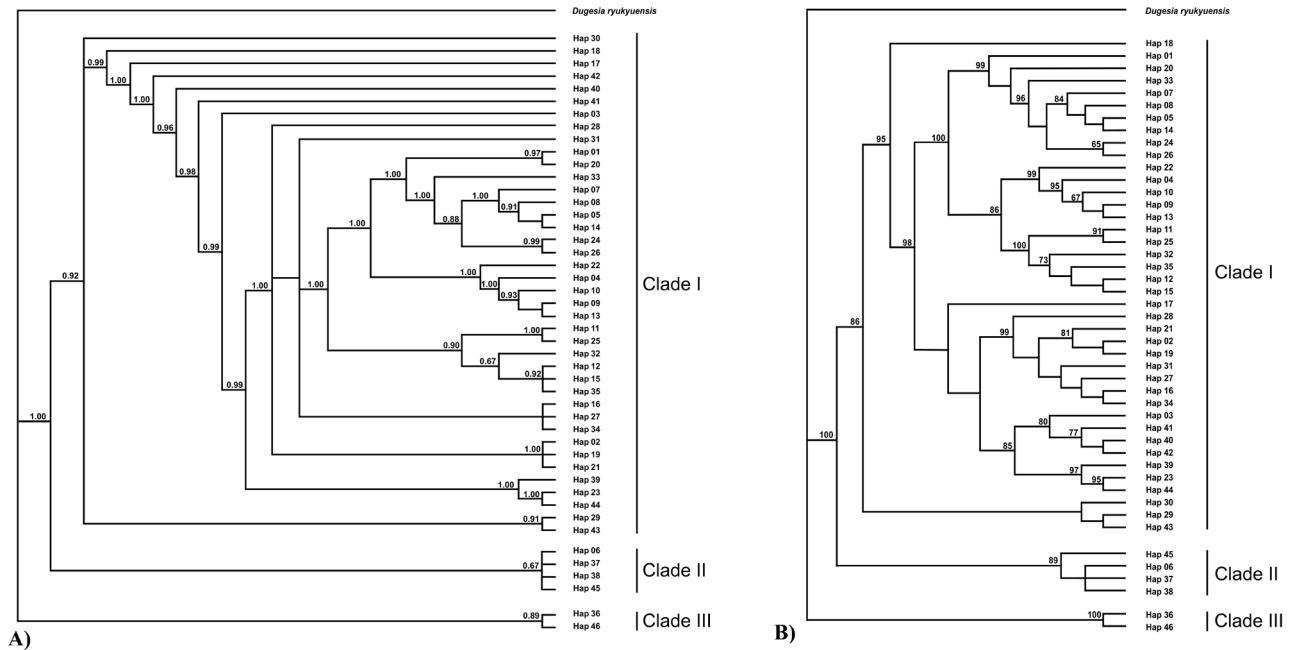Note: Shared haplotypes were underlined; * $P < 0.05$; ** $P < 0.01$.

Fig. 2. Bayesian inference (A) and maximum likelihood (B) phylogenetic trees based on the concatenated mitochondrial *Cytb* and nuclear *ITS-1* sequences. The number at the node denotes the Bayesian posterior probability (0.60 and greater only) and bootstrap percentage of maximum-likelihood analysis (60% and greater only). *Dugesia ryukyuensis* is used as the outgroup.

Table III. Analysis of molecular variance (AMOVA) of *Dugesia japonica* populations based on *Cytb-ITS*.

Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation
Among groups	1	423.681	3.034Va	3.94
Among populations within groups	18	3807.533	28.145 Vb	36.54
Within populations	100	4584.394	45.844Vc	59.52
Total	119	8815.608	77.024	

Fixation Index: F_{ST} = 0.405 (*P*<0.01).

Phylogeny and haplotype network

Using *D. ryukyuensis* (GenBank Accession number: AB618488 for *Cytb* and FJ646909 for *ITS-1*), the sister species of *D. japonica* as the outgroup, phylogenetic trees based on Bayesian inference (BI) and maximum-likelihood (ML) methods were reconstructed (Fig. 2). Both trees presented the similar topology, and all the haplotypes were clustered into three haplogroups. Most of the haplotypes clustered into Clade I. Clade II included four haplotypes (H6, H37, H38 and H45) from LaiS, Qi, LQ, LC and ZQ, and clade III consisted of only two haplotypes (H36 and H46) from WT and LC, respectively. Meanwhile, the median-joining network of all haplotypes revealed the genetic structure congruent with the phylogenetic analysis

(Fig. 3). Three haplogroups including the same haplotypes as those in the phylogenetic tree were obviously clustered.
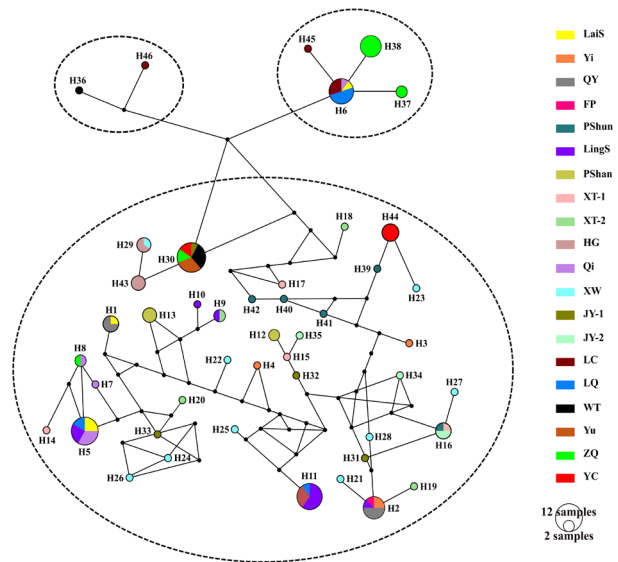

Fig. 3. Median-joining haplotype network based on the concatenated mitochondrial *Cytb* and nuclear *ITS-1* sequences. Each circle represents a specific haplotype, and its size proportional to its frequency. Different geographical populations are shown in different colors.

Table IV. Pairwise *Fst* values among *Dugesia japonica* populations.

	QY	Yi	LaiS	LingS	PShan	WT	FP	LQ	Yu	Qi	XT-1	XT-2	XW	JY-1	JY-2	ZQ	PShun	HG	YC	LC
QY	0.000																			
Yi	-0.029	0.000																		
LaiS	0.209	0.463*	0.000																	
LingS	0.293*	0.420*	0.268*	0.000																
PShan	0.415*	0.532*	0.521*	0.055	0.000															
WT	0.327*	0.325*	0.505*	0.638*	0.713*	0.000														
FP	0.155	0.212	0.392*	-0.022	0.202	0.547*	0.000													
LQ	0.117	0.226	0.165	0.392*	0.523*	0.141*	0.333	0.000												
Yu	0.409*	0.538*	0.692*	0.720*	0.917*	-0.044	0.761*	0.146	0.000											
Qi	0.343*	0.584*	-0.163	0.328*	0.606*	0.618*	0.506*	0.302*	0.784*	0.000										
XT-1	-0.130	-0.075	0.066	0.140	0.312*	0.267*	-0.021	-0.003	0.403*	0.234*	0.000									
XT-2	-0.136	-0.070	0.067	0.167	0.280*	0.222*	0.042	-0.034	0.345*	0.233	-0.239	0.000								
XW	-0.072	-0.073	0.181	0.203*	0.298*	0.281*	0.058	0.058	0.321	0.290*	-0.174	-0.160	0.000							
JY-1	-0.127	-0.042	0.057	0.139	0.311*	0.211	-0.016	-0.077	0.338	0.216*	-0.293	-0.266	-0.180	0.000						
JY-2	0.007	-0.152	0.515*	0.449*	0.614*	0.375*	0.224	0.268	0.625	0.628*	-0.055	0.016	-0.029	-0.037	0.000					
ZQ	0.454*	0.562*	0.649*	0.712*	0.845*	0.126	0.738*	0.116	0.041	0.724*	0.454*	0.405*	0.375*	0.387*	0.613*	0.000				
PShun	0.327*	0.083	0.724*	0.675*	0.840*	0.466*	0.648*	0.443*	0.822*	0.793*	0.301*	0.328*	0.228*	0.350*	0.311*	0.695*	0.000			
HG	0.448*	0.586*	0.723*	0.735*	0.925*	0.016	0.789*	0.187*	0.859*	0.802*	0.459*	0.403*	0.356*	0.396*	0.665*	0.078	0.842*	0.000		
YC	0.349	0.217*	0.682*	0.673*	0.808*	0.340*	0.649*	0.350*	0.583*	0.753*	0.325*	0.314*	0.237*	0.331*	0.385*	0.537*	0.159	0.620*	0.000	
LC	0.330*	0.329*	0.502*	0.638*	0.711*	0.126	0.546*	0.126	0.005	0.616*	0.270*	0.227*	0.285*	0.216*	0.379*	0.095	0.472*	0.060*	0.355*	0.000

Note: *$P<0.05$; The highest and lowest values are highlighted in bold.
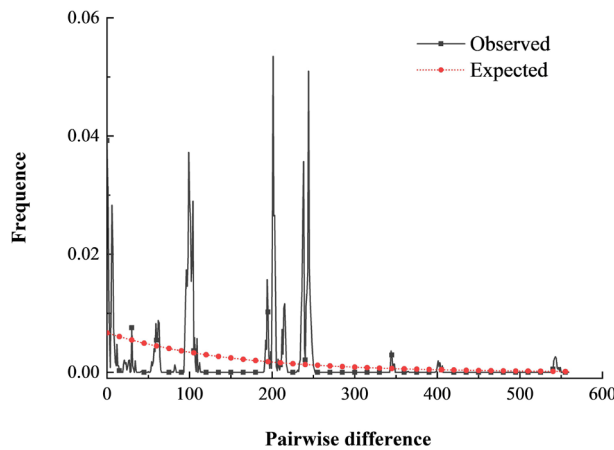

Fig. 4. Mismatch distribution based on the concatenated mitochondrial *Cytb* and nuclear *ITS-1* sequences.

Demographic history

Neutrality tests were conducted using Tajima's *D* and Fu's *Fs* statistics, and similar results were obtained for 20 geographical populations as a whole or separately. When the 20 populations were considered as one group, the Tajima's *D* and Fu's *Fs* test values were 0.250 and 1.657, respectively. As for the single population, both Tajima's *D* and Fu's *Fs* results were found positive in population QY, PShan, XT1, XT2, XW, JY1, LQ, HG and YC (Table II). Furthermore, the mismatch distribution over all populations was not distinctly unimodal but multimodal (Fig. 4).

DISCUSSION

Genetic diversity of species is the product of long-term evolution of organisms, and the level of genetic diversity is closely related to the viability and evolution potential of species (Humphries *et al.*, 1995). Research on the level, formation mechanism and distribution pattern of species genetic diversity not only can be helpful to understand the evolutionary history of species, but also can provide an important basis for analyzing the evolutionary potential of species and predicting their development trends. Haplotype diversity (Hd) and nucleotide diversity (π) are two important indicators to measure genetic diversity (Féral, 2002). As far as *D. japonica* in Taihang Mountains are concerned, the overall haplotype diversity was 0.961 and the nucleotide diversity was 0.00157, which shows the high Hd and low π pattern typical for migratory species. Low nucleotide diversity together with high haplotype diversity might be indicative of genetic

bottleneck events (Wei *et al.*, 2013; Sun *et al.*, 2015). Both the phylogenetic tree and the haplotype network diagram showed the similar results. The haplotype topology did not display the distance relationship corresponding to the geographic location of the population, which shows there is no obvious pedigree geographic pattern.

Studies on the population genetic structure not only can evaluate the variation level of populations and the relationship between different geographical groups, but also can determine the evolutionary significant units and management units so as to formulate the conservation and management strategies for resources (O'brien, 1994). Population genetic differentiation index (F_{ST}) is an important indicator to reflect the degree of genetic variation among populations (Allendorf, 1983). The degree of genetic differentiation is positively related to the F_{ST} value, and it has been suggested that a value between 0.15 and 0.25 means great differentiation and values above 0.25 very great genetic differentiation (Wright, 1978; Balloux and Lugon-Moulin, 2002). In the current study, the F_{ST} value drawn from AMOVA analysis was 0.405 ($P<0.01$) indicating very great genetic differentiation of overall *D. japonica* in Taihang Mountains. Consistently, most of the pariwise F_{ST} values showed above 0.15 (122 out of the total 190), which suggests great variation between colonies. This result also sheds light on the reason behind the haplotype composition pattern of *D. japonica* in Taihang Mountains (less shared haplotypes and more private ones). Geographical isolation would separate populations, impede gene flow, hence generate genetic differentiation (Pyron and Burbrink, 2010; Liu *et al.*, 2018). Since Cenozoic, Taihang Mountains have experienced multiple stages of geological movements and formed highly heterogeneous characteristics with a large variety of topography, soil, climate and vegetation (Geng *et al.*, 2019). The unique and complex terrain may hinder genetic communication between populations, thereby promote the high differentiation of population genes (Liu *et al.*, 2014). In addition, species with strong dispersal potential usually exhibits lower genetic variance between its different geographical populations. The limnetic *D. japonica* as a part of the benthos are slow-moving worms, which renders their long distance dispersal unlikely (Knakievicz, 2014). Therefore, the weak dispersal ability would be another reason responsible for the great genetic differentiation of *D. japonica* in Taihang Mountains.

Positive Tajima's *D* values indicate that populations have experienced a contraction while negative Tajima's *D* values occur in colonies that are undergoing demographic expansion or mutational selection (Tajima, 1989). When the 20 populations in Taihang Mountains were considered as one group, neutrality test results including Tajima's *D*

and Fu's *Fs* were both positive for the combined *Cytb/ITS-1* sequences. Furthermore, the mismatch distribution over all populations was deemed multimodal and rejected the hypothesis of a sudden expansion (Rogers and Harpending, 1992). As a result, we can speculate that *D. japonica* in Taihang Mountains should have been experiencing population depression, even though the decline might be not very notable. This has also been verified by the analysis based on mitochondrial *COI* (unpublished data). Factors such as regional climate types, topography, natural environment and human activities will affect species diversity and population richness (Meng *et al.*, 2017). *D. japonica* as a freshwater invertebrate mainly inhabits in the limnetic environment such as rivers, streams and springs. Taihang Mountains is the demarcation line in North China and has become one of the important biodiversity centers in China (Geng *et al.*, 2019). But recently, with the rapid development of mountain tourism, the aquatic environment in which freshwater planarians inhabit has been polluted or at least interfered by human activity. Additionally, water drying up in the context of global warming might be another direct reason for the population degradation of *D. japonica* in Taihang Mountains.

CONCLUSIONS

In this study, the population genetic diversity, genetic structure, phylogeography and population dynamics of freshwater *D. japonica* in Taihang Mountains were investigated. On the whole, the genetic diversity analysis presents a high Hd and low π pattern typical for migratory species. Isolation effect exerted by the special topography in Taihang Mountains and weak dispersal ability of benthonic freshwater planarians are probably main reasons for the very great genetic differentiation of *D. japonica* in Taihang Mountains. The positive neutrality test results as well as the multimodal arrangement of mismatch distribution indicate that *D. japonica* in Taihang Mountains might have been undergoing population decline. Therefore, practical measures such as reducing human interference should be taken to strengthen the conservation of *D. japonica* in Taihang Mountains. We hope these findings will arouse conservation and management strategy regarding freshwater planarians and contribute to the biodiversity in the long run.

ACKNOWLEDGMENTS

Statement of conflict of interest
The authors have declared no conflict of interests.

REFERENCES

Allendorf, F.W., 1983. Isolation, gene flow and genetic differentiation among populations. In: *Genetics and conservation: a reference for managing wild animal and plant populations* (eds. C.C. Schonewald-Cox, S.M. Chambers, B. MacBryde and W.L. Thomas). Menlo Park, Benjamin/Cummings.

Álvarez-presas, M., Carbayo, F., Rozas, J. and Riutort, M., 2011. Land planarians (Platyhelminthes) as a model organism for fine-scale phylogeographic studies: understanding patterns of biodiversity in the Brazilian Atlantic Forest hotspot. *J. Evol. Biol.*, **24**: 887-896. https://doi.org/10.1111/j.1420-9101.2010.02220.x

Avise, J.C., 2000. *Phylogeography: The history and formation of species*. Harvard University Press, London.

Balloux, F. and Lugon-Moulin, N., 2002. The estimation of population differentiation with microsatellite markers. *Mol. Ecol.*, **11**: 155-165. https://doi.org/10.1046/j.0962-1083.2001.01436.x

Cantatore, P., Roberti, M., Pesole, G., Ludovico, A., Milella, F., Gadaletal, M.N. and Saccone, C., 1994. Evolutionary analysis of cytochrome b sequences in some perciformes: Evidence for a slower rate of evolution than in mammals. *Mol. Biol. Evol.*, **39**: 589-597. https://doi.org/10.1007/BF00160404

Cebrià, F., Adell, T. and Saló E., 2018. Rebuilding a planarian: from early signaling to final shape. *Int. J. Dev. Biol.*, **62**: 537-550. https://doi.org/10.1387/ijdb.180042es

Darriba, D., Toboada, G.L., Doallo, R. and Posada. D., 2012. jModelTest 2: more models, new heuristics and parallel computing. *Nat. Methods*, **9**: 772. https://doi.org/10.1038/nmeth.2109

Excoffier, L., Laval, G. and Schneider, S., 2005. Arlequin (version 3.0): An integrated software package for population genetics data analysis. *Evol. Bioinform.*, **1**: 47-50. https://doi.org/10.1177/117693430500100003

Féral, J.P., 2002. How useful are the genetic markers in attempts to understand and manage marine biodiversity? *J. Exp. Mar. Biol. Ecol.*, **268**: 121-145. https://doi.org/10.1016/S0022-0981(01)00382-3

Fu, T.G., Han, L.P., Gao, H., Liang, H.Z., Li, X.R. and Liu, J.T., 2018. Pedodiversity and its controlling factors in mountain regions. A case study of Taihang Mountain, China. *Geoderma*, **310**: 230-237. https://doi.org/10.1016/j.geoderma.2017.09.027

Geng, S., Shi, P., Song, M., Zong, N., Zu, J. and Zhu W., 2019. Diversity of vegetation composition enhances ecosystem stability along elevational gradients in the Taihang Mountains. *China Ecol. Indic.*, **104**: 594-603. https://doi.org/10.1016/j.ecolind.2019.05.038

Geng, S., Shi, P., Zong, N. and Zhu, W., 2018. Using soil survey database to assess soil quality in the heterogeneous Taihang mountains. *North China Sustainab.*, **10**: 3443. https://doi.org/10.3390/su10103443

Guindon, S. and Gascuel, O., 2003. A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. *Syst. Biol.*, **52**: 696-704. https://doi.org/10.1080/10635150390235520

Hu, S., Wang, F., Zhan, C., Zhao, R., Mo, X. and Liu, L., 2019. Detecting and attributing vegetation changes in Taihang Mountain, China. *J. Mt. Sci-Engl.*, **16**: 337-350. https://doi.org/10.1007/s11629-018-4995-1

Huelsenbeck, J.P., Ronquist, F., Nielsen, R. and Bollback, J.P., 2001. Bayesian inference of phylogeny and its impact on evolutionary biology. *Science*, **294**: 2310-2314. https://doi.org/10.1126/science.1065889

Humphries, C.J., Willianms, P.H. and Vane Wright, R.I., 1995. Measuring biodiversity value for conservation. *Annu. Rev. Ecol. Syst.*, **26**: 93-111. https://doi.org/10.1146/annurev.es.26.110195.000521

Ireland, D., Bochenek, V., Chaiken, D., Rabeler, C., Onoe, S., Soni, A. and Collins, E.M.S., 2020. *Dugesia japonica* is the best suited of three planarian species for high-throughput toxicology screening. *Chemosphere*, **253**: 126718. https://doi.org/10.1016/j.chemosphere.2020.126718

Knakievicz, T., 2014. Planarians as invertebrate indicators in freshwater environmental quality: The biomarkers approach. *Ecotoxicol. Environ. Contamin.*, **9**: 1-12. https://doi.org/10.5132/eec.2014.01.001

Lazaro, E., Harrath, A.H., Stocchino, G.A., Pala, M., Baguñà, J. and Riutort, M., 2011. *Schmidtea mediterranea* phylogeography: An old species surviving on a few Mediterranean islands? *BMC Evol. Biol.*, **11**: 274-289. https://doi.org/10.1186/1471-2148-11-274

Librado, P. and Rozas, J., 2009. DnaSP v5: A software for comprehensive analysis of DNA polymorphism data. *Bioinformatics*, **25**: 1451-1452. https://doi.org/10.1093/bioinformatics/btp187

Liu, L., Hao, Z.Z., Liu, Y.Y., Wei, X.X., Cun, Y.Z. and Wang, X.Q., 2014. Phylogeography of *Pinus armandii* and its relatives: Heterogeneous contributions of geography and climate changes

to the genetic differentiation and diversification of Chinese white pines. *PLoS One*, **9**: e85920. https://doi.org/10.1371/journal.pone.0085920

Liu, Y., Qiu, Y., Wang, X., Yang, H., Hayashi, M. and Wei, C., 2018. Morphological variation, genetic differentiation and phylogeography of the East Asia cicada *Hyalessa maculaticollis* (Hemiptera: Cicadidae). *Syst. Ent.*, **43**: 308-329. https://doi.org/10.1111/syen.12276

Meng, Y., Qiu, Y. and Zhang, L., 2017. Effects of geographical distance and differences in climate and altitude on species dissimilarity of vascular plant communities in the Dulongjiang River Wtershed Area. *Biodivers. Sci.*, **25**: 1313-1320. (in Chinese). https://doi.org/10.17520/biods.2017076

Pagán, O.R., 2017. Planaria: An animal model that integrates development, regeneration and pharmacology. *Int. J. Dev. Biol.*, **61**: 519-529. https://doi.org/10.1387/ijdb.160328op

Pakendorf, B. and Stoneking, M., 2005. Mitochondrial DNA and human evolution. *Annu. Rev. Genom. Hum. Genet.*, **6**: 165-183. https://doi.org/10.1146/annurev.genom.6.080604.162249

Posada, D. and Crandall, K.A., 2001. Intraspecifc gene genealogies: Trees grafing into networks. *Trends Ecol. Evol.*, **16**: 37-45. https://doi.org/10.1016/S0169-5347(00)02026-7

Pyron, R.A. and Burbrink, F.T., 2010. Hard and soft allopatry: Physically and ecologically mediated modes of geographic speciation. *J. Biogeogr.*, **37**: 2005-2015.

Reddien, P.W. and Alvarado, A.S., 2004. Fundamentals of planarian regeneration. *Annu. Rev. Cell Dev. Biol.*, **20**: 725-757. https://doi.org/10.1146/annurev.cellbio.20.010403.095114

Reddien, P.W., 2018. The cellular and molecular basis for planarian regeneration. *Cell*, **175**: 327-345. https://doi.org/10.1016/j.cell.2018.09.021

Riesgo, A., Burke, E.A., Laumer, C. and Giribet, G., 2017. Genetic variation and geographic differentiation in the marine triclad *Bdelloura candida* (Platyhelminthes, Tricladida, Maricola), ectocommensal on the American horseshoe crab *Limulus polyphemus*. *Mar. Biol.*, **164**: 111-125. https://doi.org/10.1007/s00227-017-3132-y

Rogers, A.R. and Harpending, H., 1992. Population growth makes waves in the distribution of pairwise genetic differences. *Mol. Biol. Evol.*, **9**: 552-569.

Ronquist, F., Teslenko, M., van der Mark, P., Ayres, D.L., Darling, A., Höhna, S., Larget, B., Liu, L., Suchard, M.A. and Huelsenbeck, J.P., 2012. MrBayes 3.2: Efficient Bayesian phylogenetic inference and model choice across a large model space. *Syst. Biol.*, **61**: 539-542. https://doi.org/10.1093/sysbio/sys029

Sambrook, J. and Russel, D., 2016. *Molecular cloning: A laboratory manual* 3rd edn. Coldspring Harbour Laboratory Press, New York.

Solà, E., Sluys, R., Gritzalis, K. and Riutort, M., 2013. Fluvial basin history in the northeastern Mediterranean region underlies dispersal and speciation patterns in the genus *Dugesia* (Platyhelminthes, Tricladida, Dugesiidae). *Mol. Phylogenet. Evol.*, **66**: 877-888. https://doi.org/10.1016/j.ympev.2012.11.010

Sun, J.T., Wang, M.M., Zhang, Y.K., Chapuis, M.P., Jiang, X.Y., Hu, G., Yang, X.M., Ge, C., Xue, X.F. and Hong, X.Y., 2015. Evidence for high dispersal ability and mito-nuclear discordance in the small brown planthopper, *Laodelphax striatellus*. *Sci. Rep. U.K.*, **5**: 8045. https://doi.org/10.1038/srep08045

Sunnucks, P., 2000. Efficient genetic markers for population biology. *Trends Ecol. Evol.*, **15**: 199-203. https://doi.org/10.1016/S0169-5347(00)01825-5

Tajima, F., 1989. Statistical method for testing the neutral mutation hypothesis by DNA polymorphism. *Genetics*, **123**: 585-595.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M. and Kumar S., 2011. MEGA 5: Molecular evolutionary genetic analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol. Biol. Evol.*, **28**: 2731-2739. https://doi.org/10.1093/molbev/msr121

Wei, S.J., Shi, B.C., Gong, Y.J., Jin, G.H., Chen, X.X. and Meng, X.F., 2013. Genetic structure and demographic history reveal migration of the diamondback moth *Plutella xylostella* (Lepidoptera: Plutellidae) from the Southern to Northern regions of China. *PLoS One*, **8**: e59654. https://doi.org/10.1371/journal.pone.0059654

Wright, S., 1978. *Evolution and the genetics of population: variability within and among natural populations*. University of Chicago Press, Chicago.

Wu, J.P. and Li, M.H., 2018. The use of freshwater planarians in environmental toxicology studies: Advantages and potential. *Ecotoxicol. Environ. Saf.*, **161**: 45-56. https://doi.org/10.1016/j.ecoenv.2018.05.057

Zhang, H., Hu, T., Shi, C. and Chen, G., 2019a. Molecular systematics, population genetics and phylogeography on planarians (Platyhelminths: Turbellaria): A brief review of molecular markers. *Zool. Syst.*, **44**: 177-184.

Zhang, H.C., Ma, K.X., Yang, Y.J., Shi, C.Y., Chen, G.W. and Liu, D.Z., 2018. Molecular cloning, characterization, expression and enzyme activity of catalase from planarian *Dugesia japonica* in response to environmental pollutants. *Ecotoxicol. Environ. Saf.*, **165**: 88-95. https://doi.org/10.1016/j. ecoenv.2018.08.083

Zhang, H.C., Ma, K.X., Yang, Y.J., Shi, C.Y., Chen, G.W. and Liu, D.Z., 2019b. *CuZnSOD* and *MnSOD* from freshwater planarian *Dugesia japonica*: cDNA cloning, mRNA expression and enzyme activity in response to environmental pollutants. *Aquat. Toxicol.*, **208**: 12-19. https://doi.org/10.1016/j. aquatox.2018.12.013

Zhao, H., Wang, Q.R., Fan, W. and Song, G.H., 2017. The relationship between secondary forest and environmental factors in the southern Taihang Mountains. *Sci. Rep. UK.*, **7**: 16431. https://doi. org/10.1038/s41598-017-16647-0