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ORIGINAL ARTICLE

Genetic diversity, population structure and demographic history of *Dugesia japonica* in Taihang Mountains

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Abstract The freshwater planarian *Dugesia japonica* has become a model organism in regeneration biology and toxicology due to its powerful regeneration capability and highly chemical sensitivity. Nonetheless, little is known about its evolutionary history and demographics. Taihang Mountains is the natural boundary between the Loess Plateau and the North China Plain, and is considered as one of the important priority areas for biodiversity conservation in China. In order to figure out the genetic diversity, population structure and demographic history of *D. japonica* in Taihang Mountains, a study based on the mitochondrial *COI* from 116 individuals sampled across 20 populations has been conducted. The results showed that the 116 *COI* sequences yielded 32 haplotypes, including 8 shared haplotypes and 24 private ones. The overall haplotype diversity (Hd) and nucleotide diversity (π) were 0.920 and 0.083, respectively. Even though the AMOVA results suggested that the genetic variation among populations was significant (F_{ST}=0.480, P<0.01), the phylogeny and haplotype network analysis based on 32 haplotypes revealed no obvious phylogeographic pattern. Furthermore, the significantly positive values of neutrality test (Tajima's D=2.596, P<0.05; Fu's Fs=2.769, P<0.01) together with the multimodal arrangement of mismatch distribution indicated that *D. japonica* in Taihang Mountains would have been undergoing population decline. We hope these findings will arouse conservation and management strategy regarding freshwater planarians and contribute to the biodiversity in the long run.

Key words *Dugesia japonica*, Taihang Mountains, genetic diversity, population structure, demographic history.

1 Introduction

The freshwater planarian *Dugesia japonica* Ichkikawa & kawakatsu, 1964 belongs to phylum Platyhelminthes, class Turbellaria, order Tricladida, suborder Paludicola. This species mainly distributes in the Far East including China, Russia, the Korea Peninsula and the Japanese Islands (Kawakatsu *et al*., 1995). In China, it has wide distribution and large population size from southern Taiwan, Hong Kong, Yunnan to northern Jilin. At present, *D. japonica* represents an important model organism for planarian research. Planarians possess extraordinary abilities of regeneration and shape remodeling, therefore they have been traditionally used as an ideal model system for regeneration and developmental biology (Reddien & Alvarado, 2004; Elliott & Alvarado, 2013). *D. japonica* usually serves as the model organism to carry out researches on regeneration, development, tissue homeostasis and stem cells (Wagner *et al*., 2011; Tasaki *et al*., 2016; Reddien, 2018; Levin *et al*., 2019). Besides, due to their high susceptibility to xenobiotics, ease of acquisition and low maintenance cost, *D. japonica* has also

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been an ideal test animal in toxicological research (Wu & Li, 2018; Zhang *et al.*, 2018, 2019a, 2020). With the development of molecular marker and next generation sequencing technique, planarians with wide distribution and low dispersion capacity gradually become ideal model for exploring population genetics and phylogeography, and great progress has been made on species *Schmidtea mediterranea* and *D. sicula* (Álvarez-Presas *et al*., 2011; Lázaro *et al*., 2011; Lázaro & Riutort, 2013; Álvarez-Presas & Riutort, 2014; Zhang *et al*., 2019b). Nevertheless, up to now no related work concerning the population genetics and phylogeography of *D. japonica* has been reported.

The Taihang Mountains (34°36'–40°47'N, 110°42'–116°34'E) which is the natural boundary between the Loess Plateau and the North China Plain locates on the eastern edge of the second step of Chinese topography, and is considered as one of the important priority areas for biodiversity conservation in China (MEP, China, 2011). It extends from the northeast to southwest for an area of 136,500 km^2 and stretches across four administrative regions: Beijing Municipality, Hebei, Shanxi and Henan Provinces. The elevation of Taihang Mountains range from sea level to nearly 3000 m, which decrease from the northwest to the southeast (Fu *et al*., 2018). Due to the wide range of elevation and the typical earth-rocky features, Taihang Mountains possess highly heterogeneous characteristics with a large variety of topography, soil, climate and vegetation (Zhao *et al*., 2017; Fu *et al*., 2018; Geng *et al*., 2019). Considering the unique geographical position and special topography, we speculate that *D. japonica* populations in Taihang Mountains would have higher genetic differentiation and show a certain geographical pattern. Additionally, with the development and prosperity of tourism in Taihang mountain area, we wonder whether the human activity would affect the population structure and population dynamics of *D. japonica*.

Cytochrome c oxidase subunit I gene (*COI*), which has been used as the DNA barcode for rapid and accurate identification of species, locates on the mitochondria and is maternal inheritage (Hebert *et al*., 2003; Tautz *et al*., 2003). Compared with nuclear genes, its evolution rate is relatively fast and many different haplotypes typically coexist within a species. It has been proved that all of the *COI* haplotypes are geographically structured in general, and closely related haplotypes are normally geographically proximate (Avise, 2009). At present, *COI* has become a widely accepted marker for population genetics and phylogeographic study in planarians (Álvarez-presas *et al*., 2011; Lázaro *et al*., 2011; Lázaro & Riutort, 2013; Álvarea-Presas & Riutort, 2014). Therefore, in this study the genetic diversity, genetic structure, population history and phylogeographic pattern of *D. japonica* in Taihang Mountains were explored based on the molecular marker *COI* to address the aforementioned questions.

2 Materials and methods

2.1 Sample collection

Dugesia japonica samples were collected in Taihang mountains region during 2018–2019 (Fig. 1, Table S1). 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. If there were no sexually mature adults in a certain population, the juveniles were brought back indoors and reared to adulthood to guarantee reliable species identification.

2.2 DNA extraction, PCR and sequencing

High molecular weight DNA was purified from live or ethanol-fixed specimens individually using the traditional phenol-chloroform-isoamylalcohol (PCI) extraction (Sambrook *et al*., 1989) or the DNeasy Blood and Tissue Kit (Qiagen, Germany) following the manufacturer's protocol. A 1072 bp fragment of *COI* was amplified by PCR for all samples. The primer pair (COIF: 5'-GATATGGCTTTTCCTCGTGCT-3' and COIR: 5'-CAGCATAATCACAAATTCGACG-3') was designed according to the published complete mitochondrial genome of *D. japonica* using Primer premier 5.0 software (http://www.prmierbiosoft.com). The PCR was performed with a total reaction volume of 30 μL containing 15 μL TaqMix (dNTPs and Taq DNA polymerase plus, Vazyme Biotech, Nanjing, China), 12 μL ddH_2O, 1 μL 10 μM forward and reverse primers (synthesized by Genewiz Biotech, Beijing, China) and 1 μL DNA template in a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, USA). The thermocycling procedure consisted of pre-denaturation at 94℃ for 5 min, 34 cycles of denaturation at 94℃ for 40 s, annealing at 57℃ for 30 s, and extension at 72℃ for 1 min, followed by the final extension at 72℃ for 10 min. Amplification products were sequenced directly with the same amplifying primers for both reactions using ABI Prism 3730 automated sequencer (Applied Biosystem, USA).

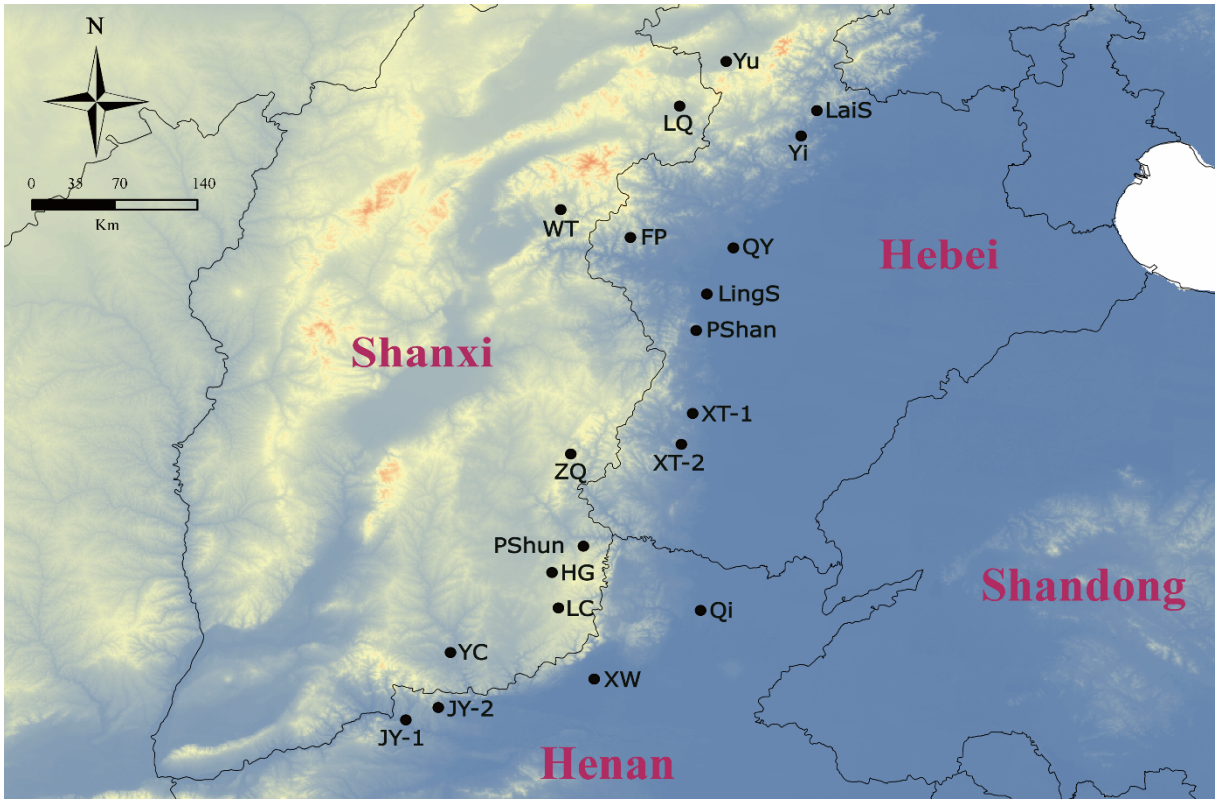

Figure 1. Geographical distribution of *Dugesia japonica* populations sampled in Taihang Mountains.

2.3 DNA Sequence analysis

Chromatograms including sense and antisense were checked manually and assembled using Seqman (DNASTAR v7.1) to obtain single consensus sequences. The accuracy of the *COI* sequences was confirmed by translating the nucleotide data into amino acid sequences by online software EMBOSS Transeq (https://www.ebi.ac.uk/Tools/st/emboss_transeq/). Multiple alignments of sequence assemblies were performed with MEGA7 (Tamura *et al*., 2011) and edited by trimming the ends so that all alignments spanned the same length. All sequences were deposited in the GenBank.

Genetic diversity including number of haplotypes (N), haplotype diversity (Hd), and nucleotide diversity (π) was computed using DnaSP v5.10.01 (Librado & Rozas, 2009). Two different approaches were both executed for demographic history analysis in DnaSP. Firstly, Tajima's *D* and Fu's *Fs* statistics were used to test for neutrality. Then, the mismatch distribution was plotted. Pairwise F_{ST} to assess the genetic differentiation between any two populations, and analysis of molecular variance (AMOVA) were calculated using the software Arlequin v3.0 (Excoffier *et al*., 2005). For the AMOVA, the sampling populations were defined as two groups (Southern and Northern groups) based on geographic distribution.

To elucidate phylogentic relationships, maximum-likelihood (ML) analysis using RAxML v8.0 (Stamatakis, 2014) and Bayesian inference (BI) by MrBayes v3.1.2 (Ronquist & Huelsenbeck, 2003) were conducted based on the best-fit nucleotide substitution model TVM for the dataset selected by Akaike Information Criterion (AIC) in the program jModeltest v2.1.7 (Darriba *et al*., 2012). Nodal support in the ML tree was evaluated by bootstrap analysis with 1000 replicates, while in the BI tree it was indicated by the posterior probability (PP) (Huelsenbeck *et al*., 2001). Tree files were visualized using FigTree v1.3 (Rambaut, 2014). A median-joining network was constructed to illustrate the evolutionary relationships among haplotypes using Network v4.6.3 under default settings (Posada & Crandall, 2001).

3 Results

We obtained 116 mitochondrial *COI* sequences (GenBank accession numbers: MT591108–MT591223) in this study. After alignment and trimming, the final length of the *COI* sequences used for subsequent analyses was 791 bp. Altogether,

the 116 sequences from Taihang Mountains yielded 32 haplotypes, including 8 shared haplotypes and 24 private ones. The overall haplotype diversity (Hd) and nucleotide diversity (π) was 0.920 and 0.083, respectively. The Hd of 20 populations represented by four or more sequences ranged from 0.000 to 1.000. Nucleotide diversity (π) was between 0.000 and 0.112 with population FP, Qi and Yu showing the lowest value while XT2 the highest (Table 1). As for neutrality tests, significantly positive Tajima's *D* and Fu's *Fs* values (2.596, $P<0.05$; 2.769, $P<0.01$) were found when 20 populations were considered as a whole and in 13 single populations, especially in population QY (2.394, $P<0.01$; 2.081, $P<0.01$), PShan (2.383, $P<0.01$; 2.070, $P<0.01$), JY1(2.326, $P<0.01$; 2.495, $P<0.01$), and YC(1.980, $P<0.05$; 1.861, $P<0.01$) (Table 1). In addition, the mismatch distribution over all populations was not unimodal but distinctly multimodal (Fig. 2).

The variation coefficient (F_{ST}) of *D. japonica* in Taihang Mountains analyzed by AMOVA was 0.480, and the statistical difference is extremely significant ($P<0.01$). The genetic variation of *D. japonica* among different populations was 49.34% while that within populations was 52.03% (Table 2). The population differentiation between any two different populations was estimated, and F_{ST} values indicated substantial genetic structuring among them. The pairwise F_{ST} values based on mitochondrial *COI* were in the range of -0.2632 to 1.0000, and showed significant differentiation in 106 of total 190 population pairs. The highest differentiation was found between population Yu and FP (1.0000, $P<0.05$) and the lowest was between population QY and JY1 (-0.2632) (Table 3).

The ML phylogenetic analysis revealed that all 32 haplotypes formed two distinct haplogroups HG1 and HG2 with robust support (Fig. 3). The HG1 branch with 100% support rate was divided into two clades. Clade 1 included 11 haplotypes and Clade 2 consisted of 10. The other branch HG2 with 99% BP value also contained two well differentiated clades (Clade 3 and 4). Clade 3 was comprised of 6 haplotypes, and Clade 4 was represented by 5 haplotypes. The BI tree also displayed two robust haplogroups with four clades, which was consistent with the ML tree in topology. The median-joining haplotype network generated similar lineage relationships to the phylogenetic analyses (Fig. 4). Four clades including the same haplotypes as those in the phylogenetic tree were uncovered. Of particular note is Clade 4. Haplotypes of Clade 4 formed a 'starburst' genealogy with H2 being in the core, and all haplotypes were connected to one another by no more than two nucleotide mutations. This result demonstrated that H2 was the ancestral haplotype and the remaining H12, 13, 19 and 26 were recently derived by separate mutations.

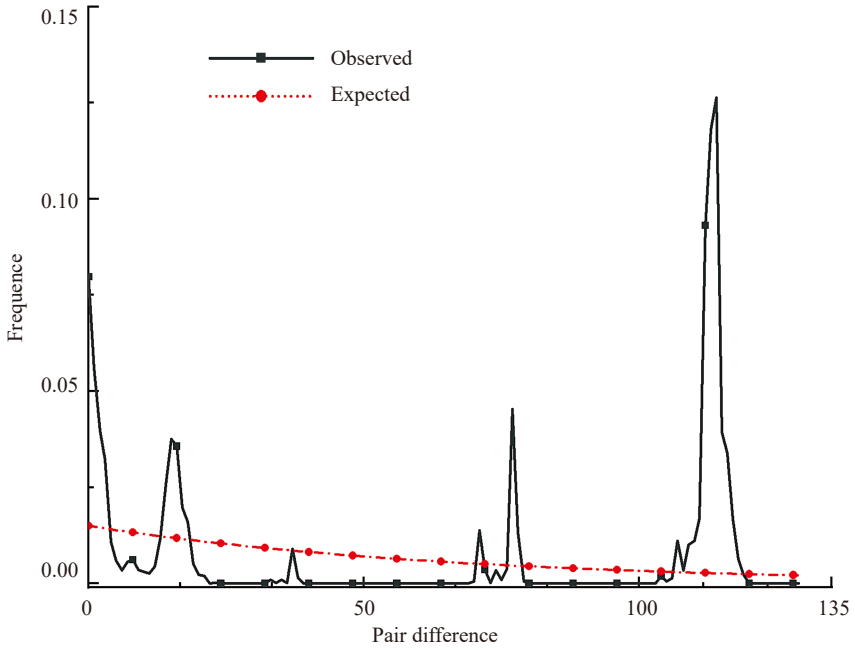

Figure 2. Mismatch distribution of *Dugesia japonica* from Taihang Mountains based on mitochondrial *COI*.

4 Discussion

Genetic diversity is the gene variation of a species in the process of its adapting to the environment, which reflects the evolutionary potential of the species (Humphries *et al*., 1995). Haplotype diversity (Hd) and nucleotide diversity (π) are two

Table 1. Genetic diversity and neutral test statistics of *D. japonica* in Taihang Mountains based on *COI*.

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	4	H4(1), H5(2), H6(1), H7(1)	0.900	0.011	1.422	1.515
Yi	4	2	H2(3), H3(1)	0.500	0.047	-0.870	-0.929
QY	6	2	H1(3), H2(3)	0.600	0.085	2.394 **	2.081**
FP	5	1	H2(5)	0.000	0.000	0.000	0.000
LingS	9	5	H2(3), H9(3), H10(1), H11(1), H12(1)	0.833	0.093	1.305	1.537
PShan	6	2	H13(3), H14(3)	0.600	0.055	2.383**	2.070**
XT1	6	4	H2(2), H6(2), H10(1), H15(1)	0.867	0.101	0.928	0.921
XT2	4	3	H1(2), H2(1), H10(1)	0.833	0.112	0.323	0.543
Qi	6	2	H6(5), H8(1)	0.333	0.000	-0.933	-0.965
XW	6	4	H6(1), H16(2), H17(1), H18(2)	0.867	0.081	0.134	1.810**
JY1	4	2	H1(2), H2(2)	0.667	0.094	2.326**	2.495**
JY2	5	2	H2(3), H19(2)	0.600	0.001	1.225	1.157
LQ	7	4	H2(1), H9(4), H23(1), H24(1)	0.714	0.042	-1.706*	-1.975**
WT	4	4	H1(1), H20(1), H21(1), H22(1)	1.000	0.007	0.083	0.083
Yu	5	1	H1(5)	0.000	0.000	0.000	0.000
ZQ	11	4	H1(3), H6(1), H7(4), H25(3)	0.782	0.009	0.853	1.133
PShun	5	2	H10(4), H26(1)	0.400	0.039	-1.264	-1.373**
HG	5	2	H27(3), H28(2)	0.600	0.001	1.225	1.157
LC	5	3	H6(1), H31(3), H32(1)	0.700	0.002	-1.048	-1.052
YC	8	5	H1(1), H16(4), H18(1), H29(1), H30(1)	0.786	0.076	1.980*	1.861**
Total	116	32		0.920	0.083	2.596*	2.769**

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

important indices indicating the genetic diversity of a species. In the current study, the Hd of the 116 *COI* sequences was 0.920 while the π value equaled 0.083. The high Hd level and low π level, a finding that is typical for migratory species, disclose that different geographic colonies of *D. japonica* in Taihang Mountains might experience the bottleneck effect (Wei *et al*., 2013; Sun *et al*., 2015). The genetic differentiation coefficient F_{ST} is usually used to measure the genetic variance degree among different populations. In the range of 0 to 1, the greater the F_{ST} value is, the higher the degree of genetic differentiation is. A value lying in the range of 0 to 0.05 indicates little genetic differentiation; a value between 0.05 and 0.15 suggests moderate differentiation; a value in the range 0.15–0.25, great differentiation; and values above 0.25 mean very great genetic differentiation (Wright, 1978; Balloux & Lugon-Moulin, 2002). In this study, the F_{ST} of overall *D. japonica* in Taihang Mountains was 0.480 (P<0.01), suggesting the genetic differentiation of populations was significant. Also, most of the pairwise F_{ST} showed significant or extremely significant difference (P<0.05 or P<0.01). Species with strong dispersal potential usually exhibits lower genetic variance between its different populations (Wang & Zhou, 2016). *D. japonica* move slowly in the freshwater environment where they inhabit and so have weak dispersal ability. In addition, the mountainous area's special topography has also exerted the isolation effect and therefore increased the genetic variation between different colonies.

Even though the phylogeny and network of *COI* haplotypes suggested that the 32 haplotypes were divided into two groups, these haplotypes dispersed in different populations and revealed no obvious phylogeographic pattern. A star phylogeny generally indicates an expanding population after experiencing the population bottlenecks (Slatkin & Hudson, 1991). The median-joining network based on *COI* haplotypes of *D. japonica* in Taihang Mountains was not typical starlike. This suggests *D. japonica* in Taihang Mountains has not been undergoing population expansion. Tajima's *D* is indistinguishable from zero if a population is selectively neutral and at equilibrium. 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). In the current study, significantly positive Tajima's *D* and Fu's *Fs* values were found in *COI* sequences as a whole (2.596, P<0.05; 2.769, P<0.01). Furthermore, the mismatch distribution over all populations was deemed multimodal and rejected the hypothesis of a sudden expansion (Rogers & Harpending, 1992). As a result, we can speculate that *D. japonica* in Taihang Mountains should have been experiencing population depression. This has also been verified by the analysis based on concatenated mito-nuclear genes (unpublished

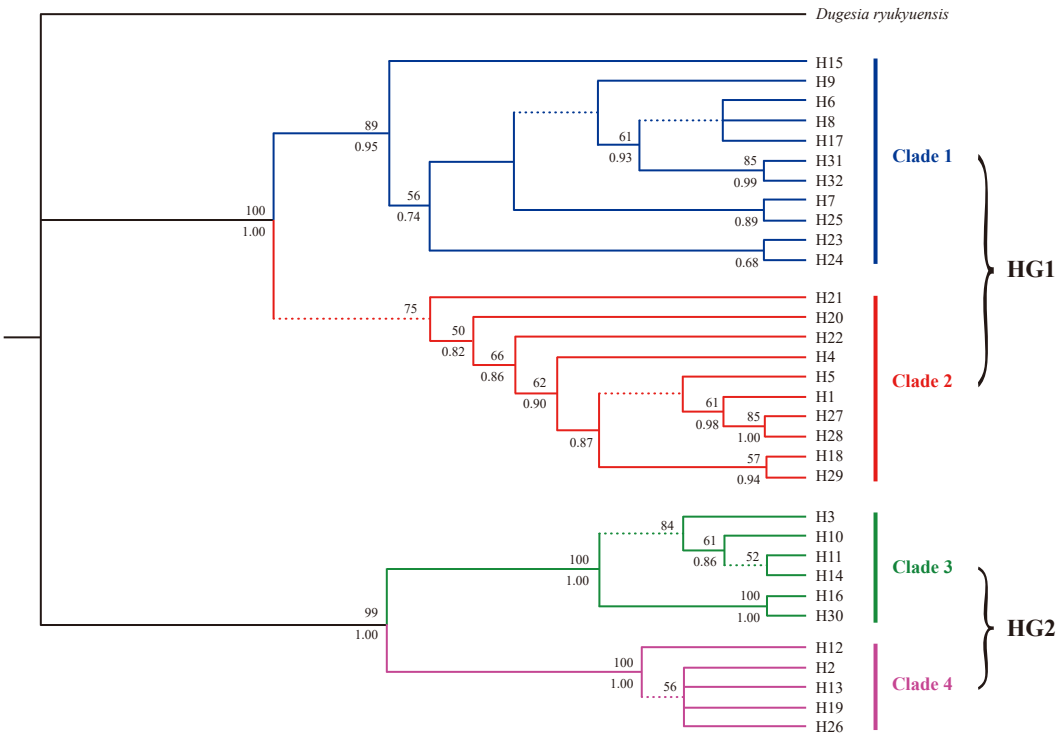

Figure 3. Maximum likelihood (ML) and Bayesian inference (BI) phylogentic trees based on mitochondrial gene *COI*. *Dugesia ryukyuensis* (Genbank accession no. AB618488) serves as the outgroup. The broken lines denote inconsistent branches. Bootstrap percentages (BP, >50 only) of ML analysis and posterior probabilities (PP, >0.50 only) of Bayesian inference are shown above and below the branch, respectively. HG—haplogroup.

data). The Taihang Mountain is the demarcation line in North China (Geng *et al*., 2019), and has been one of the important biodiversity centers in China. But in recent years with the rapid development of mountain tourism, many scenic spots have been developed, and the natural habitats of freshwater planarians (springs, brooks, etc.) have been polluted or at least been interfered. Additionally, global warming and water drying up might be another reason responsible for the population degradation of freshwater *D. japonica* in Taihang Mountains.

Table 2. Analysis of molecular variance (AMOVA) of *D. japonica* populations based on *COI*.

Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation	F statistics
Among groups	1	94.483	-0.457Va	-1.37	F_{CT}=-0.014
Among populations within groups	18	2015.060	46.465 Vb	49.34	F_{SC}=0.487**
Within populations	96	1666.983	17.364 Vc	52.03	F_{ST}=0.480**
Total	115	3776.526	33.372		

Note: *P<0.05; **P<0.01.

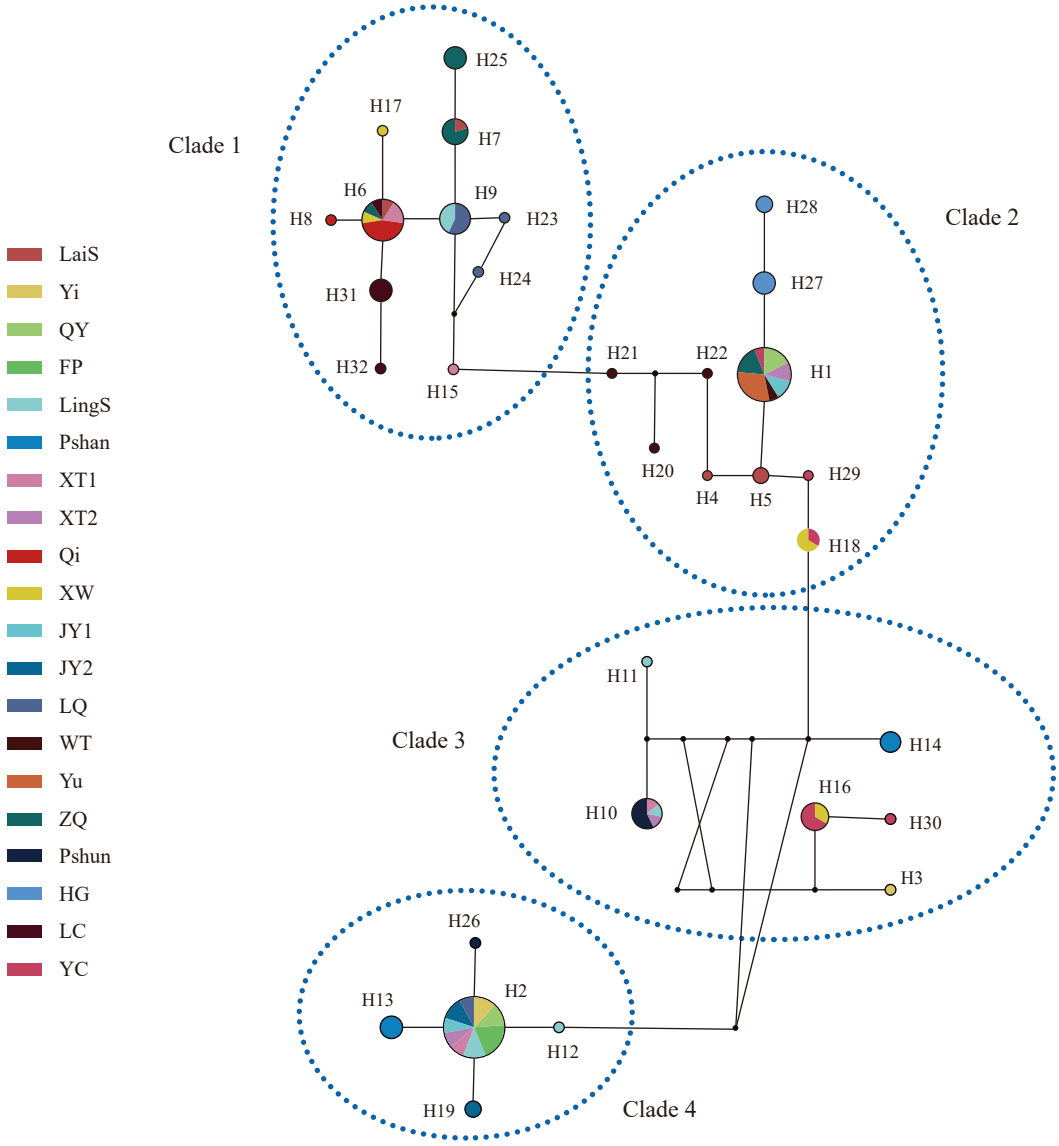

Figure 4. Median-joining haplotype network based on mitochondrial gene *COI*. The four ellipses represent four clades in Figure 3, respectively. Each circle represents a haplotype, the area of the circle is proportional to the frequency of haplotypes, and black dots represent hypothetical unobserved haplotypes. Different populations are shown in different colors.

Table 3. Pairwise genetic differences (Fst) value between *D. japonica* populations.

	LaiS	Yi	QY	LQ	WT	Yu	FP	LingS	PShan	JY1	JY2	PShun	HG	LC	YC	Qi	XW	ZQ	XT1	XT2
LaiS	0.0000																			
Yi	0.8134[a]	0.0000																		
QY	0.3370[a]	0.1720	0.0000																	
LQ	0.0752	0.6538[a]	0.2124	0.0000																
WT	-0.0565	0.8107[a]	0.3055	0.1394	0.0000															
Yu	0.3677[b]	0.8561[b]	0.3617	0.3557[b]	0.4779[a]	0.0000														
FP	0.9620[b]	0.0625	0.3617	0.8008[a]	0.9790[b]	**1.0000**[b]	0.0000													
LingS	0.3949[b]	0.0241	-0.0528	0.2497	0.3799[b]	0.4461[b]	0.2239	0.0000												
PShan	0.7504[b]	-0.0418	0.2412	0.6248[b]	0.7435[b]	0.7840[b]	0.3617	0.0585	0.0000											
JY1	0.3532	0.1427	**-0.2632**	0.1955	0.3163	0.3939	0.3939	-0.1051	0.2176	0.0000										
JY2	0.9593[b]	0.0657	0.3613	0.7977[b]	0.9760[b]	0.9973[b]	0.2500	0.2215[b]	0.3610	0.3924	0.0000									
PShun	0.8268[b]	0.3768	0.4294[b]	0.7016[b]	0.8260[b]	0.8644[b]	0.7476	0.2152	0.1740	0.4242[b]	0.7444	0.0000								
HG	0.5000[b]	0.8555[b]	0.3823	0.3945[b]	0.6110[b]	0.8750[b]	0.9974[b]	0.4581[b]	0.7862[b]	0.4144[a]	0.9948[a]	0.8646[b]	0.0000							
LC	0.5446[b]	0.8521[b]	0.4451[a]	0.0501	0.7578[b]	0.9655[b]	0.9948[a]	0.4230[b]	0.7840[a]	0.4783[b]	0.9921[b]	0.8604[b]	0.9546[b]	0.0000						
YC	0.4693[b]	0.3821[a]	0.2143	0.3995[a]	0.4520[a]	0.5012[a]	0.6003[b]	0.1617[a]	0.3169[b]	0.1815	0.5974[b]	0.3253[b]	0.5054[a]	0.5350[b]	0.0000					
Qi	0.5577[b]	0.8723[b]	0.4805[b]	0.0333	0.7938[b]	0.9886[b]	0.9984[b]	0.4513[b]	0.8057[b]	0.5235[b]	0.9960[a]	0.8780[b]	0.9757[b]	0.6286[b]	0.5598[b]	0.0000				
XW	0.1325	0.4573[b]	0.0987	0.0690	0.1288	0.2367	0.6575[b]	0.1208	0.4219[b]	0.0550	0.6544[a]	0.4668[b]	0.2583[a]	0.2279	0.0137	0.2527[a]	0.0000			
ZQ	0.0512	0.8712[b]	0.5051[b]	0.0872[a]	0.2728	0.5787[b]	0.9570[b]	0.5086[b]	0.8213[b]	0.5468[b]	0.9554[b]	0.8756[b]	0.6358[b]	0.3783[a]	0.5934[b]	0.3170[b]	0.2854	0.0000		
XT1	0.2493	0.1696	-0.0969	0.0687	0.2347[a]	0.3411	0.3910	-0.1133	0.1912	-0.1552	0.3871	0.3018	0.3598[a]	0.2762[a]	0.1254	0.3116[a]	-0.0240	0.3893	0.0000	
XT2	0.2473	0.1749	-0.1688	0.1431	0.2065	0.2843	0.4671[a]	-0.1144	0.1630	-0.2363	0.4655[a]	0.2485	0.3093[b]	0.3818[a]	0.0302	0.4311[b]	-0.0820	0.4582	-0.1890	0.0000

Note: The highest and lowest values were highlighted in bold. [a]$P<0.05$, [b]$P<0.01$.

5 Conclusion

In the current study, the genetic diversity analysis disclosed a high Hd and low π pattern typical for migratory species. Even though the AMOVA results and pairwise F_{ST} (higher F_{ST}) suggested that the genetic variations among most populations were significant, the phylogeny analysis revealed no obvious phylogeographic pattern. The significantly positive neutrality test results and the multimodal arrangement of mismatch distribution together indicated that *D. japonica* in Taihang Mountains should have been undergoing decline. Understanding its population history is crucial in placing the current conservation plight in context. We hope these findings will arouse conservation and management strategy regarding freshwater planarians and contribute to the biodiversity in the long run.

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