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Population Genetic Structure of the Invasive Red Swamp Crayfish in China Revealed by ITS1 Variation

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Abstract The invasive red swamp crayfish (*Procambarus clarkii*) provides a valuable opportunity for studying the population genetics of invasive species that disperse rapidly. We analyzed the population genetic structure among 12 populations of the crayfish in China based on the internal transcribed spacer 1 (ITS1) region. The ITS1 of 815 bp aligned across 34 haplotypes; the average GC content was 53.9%. AMOVA showed that intrapopulation variation (95.26%) was much higher than interpopulation variation (4.74%). Genetic differentiation between the Taiwan and mainland populations ($F_{st} = 0.160$) was moderate, but the Chinese population (Taiwan and the mainland combined) and an American population were highly differentiated (0.682 and 0.977, respectively). Gene flow between the Chinese and American populations ($N_m = 0.006$ and 0.117, respectively) was lower than that between Taiwan and the mainland (1.536). Phylogenetic trees showed that three major genealogical clusters matched the sample locations well, suggesting that genetic differentiation is created largely by geographic isolation.

Keywords Red swamp crayfish · *Procambarus clarkii* · Invasive alien species · Population genetic structure · Internal transcribed spacer 1 (ITS1) region

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Introduction

Population genetic structure provides an understanding of the adaptability of a species, both potential and evolutionary, and also reflects its population history (Moreno et al. 2004; Hardesty et al. 2010). Phylogeographic studies show that habitat has a considerable influence on the population genetic structure, distribution, and evolution of many animals, especially of alien invasive species (Ellstrand and Elam 1993; Qu et al. 2005; Yang et al. 2006, 2009; Hardesty et al. 2010; Sassi et al. 2011). Recently, close attention has been paid to biological invasions, and the increasing invasive rate has become a global problem, as exotic or introduced species have often caused ecological disaster (Barbaresi et al. 2003; Yue et al. 2008, 2010). Many studies have shown that the molecular variability and population genetic structure of the keystone invasive species would change in new habitats. Comparing the genetic structure of introduced populations with native ones will increase our understanding of genetic differentiation and gene flow (Ellstrand and Elam 1993; Barbaresi et al. 2003).

The red swamp crayfish, *Procambarus clarkii*, is a temperate freshwater crayfish native to the south-central United States and northeastern Mexico (Huner 1988; Barbaresi et al. 2003). It is strongly adaptive and competitively dominant when introduced into a suitable habitat, and it can establish itself and become a keystone species within a short time (Barbaresi et al. 2003; Yue et al. 2010). Because of its economic importance, it has been introduced into many countries in the world (Yue et al. 2010). The species was introduced to Nanjing, China, from Japan in 1929. Since then, it has been spread by humans to the middle and lower reaches of the Yangtze, Huaihe, and Yellow river plains as a source of food. It has been heavily exploited as a fishery product and is now used widely in aquaculture in China (Li et al. 2005; Wang et al. 2009; Cao et al. 2010; Yue et al. 2008, 2010). Red swamp crayfish have long been recognized as an important species for biological invasion studies (Barbaresi et al. 2003). The invasive red swamp crayfish is a valuable model for studying the population genetic consequences of species that disperse rapidly.

Nuclear rDNA sequences have been widely used to estimate the molecular variability and population genetic structure of many organisms (Vogler and Desalle 1994; Miller et al. 1996; Fabry et al. 1999; Schulenburg et al. 1999). The internal transcribed spacer 1 (ITS1) region is between the 18S rDNA and 5.8S rDNA genes (or their homologs) in the nuclear rDNA sequence. It displays a relatively high level of variant nucleotide positions that are useful markers for molecular evolutionary studies. These markers are widely used for phylogenetic studies and population genetic structure analysis, especially at the population and species levels (Harris and Crandall 2000; Coleman and Vacquier 2002; Coleman 2003; Ji et al. 2003). ITS1 gene sequences within geographic populations are similar and show little variation, but between different geographic populations they display great variation (Coleman and Vacquier 2002).

The molecular variability and population genetic structure of the red swamp crayfish in different rivers and lakes in China may diversify through isolation effects; however, at present little is known about this. In this study, we present preliminary results on the molecular variability of the ITS1 region and analyze the population

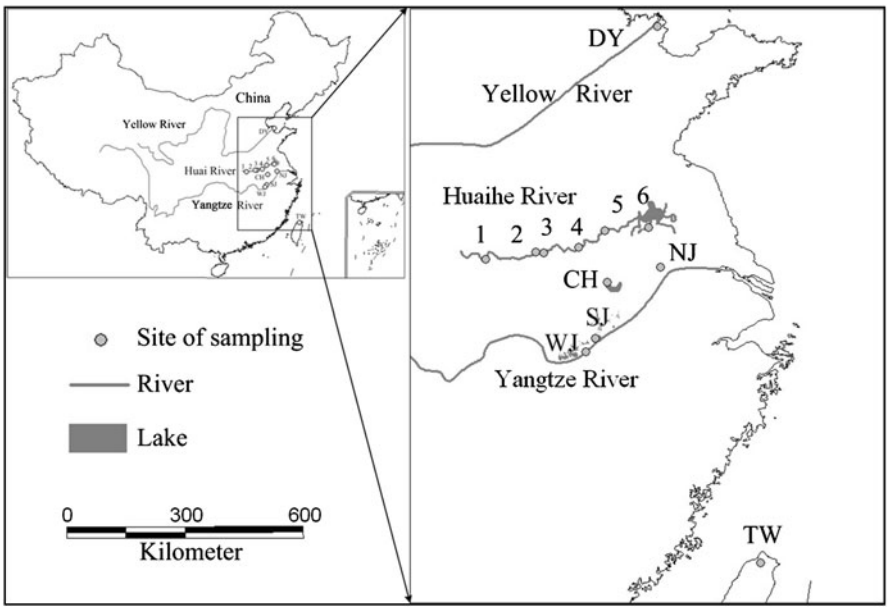

Fig. 1 Sampling locations of the red swamp crayfish used in this study, from three river systems in China Mainland and from Taiwan. Sites in the Huaihe River: *1* Gan'an, *2* Huaibin, *3* Fuyang, *4* Yingshang, *5* Bengbu, *6* Xuyi. Other population codes as in Table 1

genetic structure in different geographic populations of the red swamp crayfish in China using ITS1 markers. The aim of this study is to describe the molecular variability and population genetic structure of 12 Chinese populations of the red swamp crayfish, obtained by comparing their ITS1 sequences, and to outline potential avenues of dispersal in China.

Materials and Methods

Sample Collection

Adult red swamp crayfish were collected from the natural waters of China Mainland and Taiwan (Fig. 1). The mainland population samples were collected from 11 locations: 6 in the Huaihe River basin (Fuyang, Gan'an, Huaibin, Bengbu, Xuyi, Yingshang), 4 in the Yangtze River basin (Nanjing, Chaohu Lake, Shingjin Lake, Wangjiang), and 1 in the Yellow River delta, Dongying City, Shandong Province. Sample numbers from each of the locations ranged from 9 to 24, for a total of 196 adult individuals (Table 1). Each individual collected in the field was stored in absolute ethanol until DNA extraction. To explore the deeper phylogeographic relationships among the red swamp crayfish populations across a greater range, we collected ITS1 sequences for the species that originated in America from GenBank (accession nos. AF198585–AF198590).

Table 1 Study sites and sample size of the red swamp crayfish populations used in this study

Population (*N*)	River system	Local population	Code	Sampling site	Sample size
China Mainland (176)	Yellow River	Dongying	DY	Fuxing Village, Kenli County	18
	Huaihe River				97
		Fuyang	FY	Wangjiaba Town	22
		Gan'an	GA	Gan'an Huaihe River Bridge	10
		Huaibin	HB	Huaibin Huaihe River Bridge	15
		Xuyi	XY	Xuyi Huaihe River Bridge	14
		Bengbu	BB	Bengbu Sluice Gate	12
		Yingshang	YS	Lukou Town	24
	Yangtze River				61
		Nanjing	NJ	Xuanwu Lake	20
		Chaohu Lake	CH	Yicheng	15
		Shengjin Lake	SJ	Lianhe Conservation Station	9
		Wangjiang	WJ	Huayang River	17
Taiwan, China (20)	Taiwan	Taibei	TW	Jinlong Lake	20
American (6)		America	AM	GenBank acc. nos. AF198585–AF198590	6

DNA Extraction, PCR Amplification, and Sequencing

Total DNA was extracted from tail muscle tissue of selected samples using the proteinase K/SDS method, stored at −20°C, and used as templates in polymerase chain reactions (PCR). The ITS1 primers (5′-CACACCGCCCGTCGCTACTA-3′ and 5′-ATTTAGCTGCGGTCTTCATC-3′) were synthesized by Sangon Biotech (Shanghai) Co., Ltd. The PCR amplification was carried out in final volumes of 25 µl containing 100 ng of template DNA, 2.5 µl 10× reaction buffer, 1 µl 25 mmol/l $MgCl_2$, 2 µl 2.5 mmol/l dNTPs, 1 µl 10 µmol/l each primer, 0.5 U *Taq* DNA polymerase (Trans Taq-T DNA Polymerase), and sterile double-distilled water to make up the final volume. The following conditions were used for the PCR amplifications: denaturation for 5 min at 94°C; followed by 35 cycles of denaturation for 30 s at 94°C, annealing for 30 s at 56.8°C, elongation for 1 min at 72°C; and a final extension step at 72°C for 10 min. The PCR products were purified and sequenced by Sangon Biotech (Shanghai) Co., Ltd. All fragments were sequenced in both directions.

Sequence Alignment and Phylogenetic Analysis

The total rDNA ITS1 sequences were edited using the DNAstar program (Madison, WI, USA). Multiple sequence alignments were made with Clustal X 1.81 (Thompson et al. 1997), then refined manually and checked by visual inspection. Analysis of molecular variance (AMOVA) was performed to partition the total phenotypic variance into intra- and interpopulation variances using Arlequin 3.1

software (Excoffier et al. 2005). Genetic differentiation was analyzed by calculating pairwise fixation index (F_{st}) values among the crayfish geographic populations using Arlequin software. Gene flow (N_m) was calculated as $1 - F_{st}/4F_{st}$. Haplotypes were identified using the Dambe software package (http://en.bio-soft.net/format/dambe.html) and submitted to GenBank (acc. nos. JQ776599–JQ776632). The DnaSP software package (http://www.ub.edu/dnasp/) was used to calculate haplotype diversity (*h;* Nei 1987) and nucleotide diversity (π; Nei 1982) between populations. Variable and parsimony informative sites in the sequences were calculated using Mega 4.0 (http://www.megasoftware.net). Phylogenetic trees were constructed using neighbor-joining, maximum likelihood, and Bayesian inference algorithms. Neighbor-joining trees were also reconstructed using Mega 4.0 and assessed through bootstrapping with 1,000 replications. For the maximum likelihood analysis, the PAUP software (version 4.0b8; http://paup.csit.fsu.edu/) was used, and the bootstrap values were evaluated via the bootstrap test with 100 iterations. Bayesian inference of phylogeny was performed using MrBayes 3.1.2 (http://mrbayes.sourceforge.net/), and the analyses were run for one million generations until the average standard deviation of split frequencies was less than 0.01, meaning that convergence was reached. Chains were sampled every 1,000 generations.

Results

Sequence Variation and Haplotype Distribution

The ITS1 regions from all 196 individuals from the 12 populations were successfully amplified by PCR. The range of lengths for the ITS1 sequences was 854–881 bp. The mean total nucleotide composition of the sequences was A 24.0%, C 27.2%, G 26.7%, and T 22.1%; the average GC content (53.9%) was slightly higher than the AT content (46.1%). When the 196 sequences were aligned, the 815 bp of the ITS1 regions contained 319 variable sites and 49 parsimony informative sites.

The 196 ITS1 sequences were screened, and 34 haplotypes were identified. A few of them showed a wide geographic distribution, but most had a limited geographic distribution (Table 2). Of the 34 haplotypes, 9 (26.47%) were shared in the Taiwan population. Two haplotypes (Hap_1, Hap_3) showed a wide geographic distribution, 6 (Hap_1, Hap_2, Hap_3, Hap_5, Hap_8, and Hap_10) were shared by different populations, and the others were unique to the corresponding population (Table 2). Hap_1, scattered across the 12 sample collection sites, was present in 121 sequences (61.73%). Hap_3 was found in 9 populations (Fuyang, Gan'an, Huaibin, Xuyi, Nanjing, Chaohu Lake, Shingjin Lake, Wangjiang, and Taiwan) and was present in 31 sequences (15.82%). Hap_5, Hap_8, and Hap_10 were shared between 2 of the populations, and the other haplotypes were found in only one population.

Population Genetic Diversity

Significant divergence was observed across all samples ($F_{st} = 0.047$, $P < 0.001$; $N_m = 5.069$), indicating a low level of geographic population structure in the 12

Table 2 Shared haplotypes in the ITS1 region based on the complete data set for 12 red swamp crayfish populations in China

Haplotype	Population[a]											
	TW	DY	FY	GA	HB	XY	BB	YS	NJ	CH	SJ	WJ
Hap_1	10	11	12	5	6	8	11	18	11	11	6	12
Hap_2			1							2		
Hap_3	3		4	5	6	2			4	3	2	2
Hap_4											1	
Hap_5			4		2							
Hap_6					1							
Hap_7									1			
Hap_8						1			1			
Hap_9									1			
Hap_10								1	1			
Hap_11	1											
Hap_12	1											
Hap_13	1											
Hap_14	1											
Hap_15	1											
Hap_16	1											
Hap_17	1											
Hap_18												1
Hap_19												1
Hap_20							1					
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Hap_24			1									
Hap_25			1									
Hap_26								1				
Hap_27								1				
Hap_28								1				
Hap_29								2				
Hap_30		1										
Hap_31		1										
Hap_32		1										
Hap_33		1										
Hap_34		2										

[a] Population codes as in Table 1

populations. AMOVA indicated that a high proportion of the total genetic variance was attributable to variations within populations (95.26%), whereas only 4.74% occurred among populations (Table 3). The F_{st} values were between 0.160 and 0.977, and the N_m values were between 0.006 and 1.536 in each population (Table 4).

Table 3 Analysis of molecular variance (AMOVA) for 12 red swamp crayfish populations in China

Source of variation	Degrees of freedom	Sum of squares	Variance components	% Variation
Among populations	11	5.097	0.013	4.74
Within populations	185	47.179	0.256	95.26
Total	196	52.276	0.269	100

Table 4 Population differentiation among the red swamp crayfish populations

Population	Taiwan	China Mainland	American
Taiwan	–	1.536	0.117
China Mainland	0.160	–	0.006
American	0.682	0.977	–

Below diagonal Population differentiation (F_{st}); *above diagonal* gene flow level (N_m)

Table 5 Genetic diversity in the Chinese and American populations of red swamp crayfish

Population[a]	Diversity		Tajima's D test
	Haplotype (h)	Nucleotide (π)	
BB	0.506 ± 0.125	0.001 ± 0.0005	0.366
XY	0.681 ± 0.132	0.011 ± 0.008	0.065
FY	0.619 ± 0.077	0.003 ± 0.003	0.320
GA	0.556 ± 0.075	0.0007 ± 0.0004	0.855
HB	0.705 ± 0.074	0.001 ± 0.0007	−0.157
YS	0.442 ± 0.124	0.001 ± 0.001	0.578
NJ	0.621 ± 0.109	0.004 ± 0.003	−1.583
CH	0.419 ± 0.113	0.0005 ± 0.0003	−0.760
WJ	0.419 ± 0.141	0.0013 ± 0.0005	−1.300
DZ	0.556 ± 0.165	0.002 ± 0.001	0.097
DY	0.544 ± 0.018	0.003 ± 0.002	−2.573
ML	0.495 ± 0.041	0.003 ± 0.002	−2.573
TW	0.832 ± 0.075	0.049 ± 0.008	−2.503
AM	0.333 ± 0.046	0.0005 ± 0.0003	−0.933

[a] Population codes as in Table 1

In this study, the red swamp crayfish populations presented a high average haplotype diversity ($h = 0.561 \pm 0.001$; range 0.419–0.832) and a low average nucleotide diversity ($\pi = 0.007 \pm 0.0004$; range 0.0005–0.049). The nucleotide diversity value of 5 populations (Fuyang, Huaibin, Xuyi, Nanjing, and Taiwan) was higher than the average value for all 12 populations. The Taiwan population exhibited the greatest level of variability ($h = 0.832 \pm 0.075$, $\pi = 0.049 \pm 0.008$), whereas the lowest level of variability was for the Chaohu Lake population ($h = 0.419 \pm 0.113$, $\pi = 0.0005 \pm 0.0003$) (Table 5). In addition, the Taiwan population exhibited a greater level of genetic variability ($h = 0.832 \pm 0.075$,

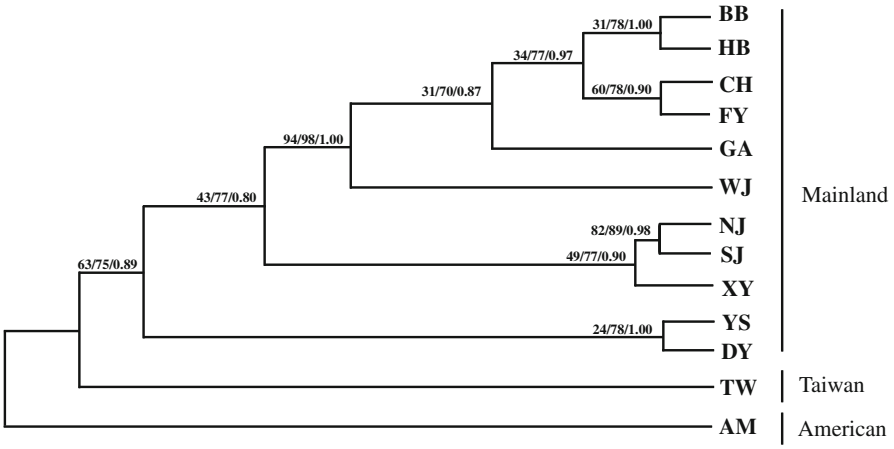

Fig. 2 Phylogenetic relationships of geographic populations of the red swamp crayfish based on Nei's genetic distance. Numbers at each node are bootstrap values from three analyses (neighbor-joining/maximum likelihood/Bayesian inference). Population codes as in Table 1

$\pi = 0.049 \pm 0.008$) than the mainland population ($h = 495 \pm 0.041$, $\pi = 0.003 \pm 0.002$).

Phylogenetic Relationships

The neighbor-joining, maximum likelihood, and Bayesian inference phylogenetic trees converge on a congruent topology, and genetically the populations cluster into three major groups (Fig. 2), with significant genealogical clusters or clades associated with the sample collecting locations. Samples from the Huaihe (Fuyang, Gan'an, Huaibin, Bengbu, Xuyi, Yingshang), Yangtze River (Nanjing, Chaohu Lake, Shingjin Lake, Wangjiang), and Yellow River formed the mainland population cluster, and the Taiwan population formed the second cluster. The Taiwan cluster formed sister groups with the mainland population, and both of them grouped together with the American population. Within the mainland population clade, some geographic populations were irregularly distributed according to the river systems, but others were well matched (e.g., Chaohu Lake, Wangjiang, Xuyi, and Yingshang).

Discussion

Characterization of the ITS1 Region and Haplotype Distribution

A better understanding of the characteristics of the ITS1 region will provide insights into the evolution dynamics of organisms. The length of the ITS1 region in the red swamp crayfish is similar to other crayfish (Harris and Crandall 2000; Campbell et al. 2005; Kan et al. 2007). The GC content has some important functional relevance for an organism's genome; for example, it is concerned with coding-sequence length and

also correlates with adapting ability (Fullerton et al. 2001; Galtier et al. 2001; Kan et al. 2007). In the ITS1 region of the red swamp crayfish, the high GC content (53.9%) is similar to other organisms (Harris and Crandall 2000; Coleman and Vacquier 2002; Kan et al. 2007). Intra-individual sequence variation in the ITS1 regions of populations from Taiwan and China Mainland was sometimes greater than interindividual variation in each population, indicating, as might be expected, that gene flow between the Taiwan and mainland populations was lower (i.e., gene exchange was restricted) than between individuals in the same population.

Comparisons of the haplotypes revealed contrasting patterns in the 12 population structures. Hap_1 was found in all geographic populations, suggesting that it is relatively conservative, primordial, and stable, having adapted strongly to the environment in the history of evolution. Haplotype variability and molecular diversity was greatest in the Taiwan population (9 shared haplotypes) compared with the others, showing that the genetic variability at the population level was not high. This finding is similar to the results of a microsatellite analysis reported previously (Yue et al. 2010).

Population Genetic Structure

The ability of a population of a species to adapt to the habitat depends mainly on its genetic diversity and genetic structure (Bazin et al. 2006). Of the total genetic diversity in the red swamp crayfish populations, 95.26% was attributable to within-population diversity and the remainder (4.47%) to diversity among populations. This finding indicated that at low levels of geographic population structure, there was only a small differentiation among the 12 populations; however, high migration and breeding rates in red swamp crayfish overcome low genetic diversity, suggesting that most populations of this species still maintain considerable genetic diversity (Saccheri et al. 1998; Nichols et al. 2003; Yue et al. 2010).

Significant F_{st} values across all the samples from the Taiwan and mainland populations were observed. When we compared our Chinese samples from the Taiwan and mainland populations ($F_{st} = 0.682$) with the American population (0.977) of red swamp crayfish, we found that the F_{st} values were highly differentiated, suggesting that the genetic difference between these two populations was remarkable. Conversely, the genetic difference between the Taiwan and mainland populations was moderate ($F_{st} = 0.160$). The population pairwise gene flow values between the Chinese and American populations ranged from 0.006 to 1.536, showing that the gene flow was quite low ($N_m = 0.006$ and 0.117, respectively), similar to the genetic structure reported in European populations (Barbaresi et al. 2003). Gene flow between the Taiwan and mainland populations was also not high in our samples ($N_m = 1.536$), possibly because of the long geological isolation of these crayfish. Low genetic diversity as a result of the founder effect should be expected for the settled populations, together with a high population differentiation (Barbaresi et al. 2003). A new population may be distinctive, both genetically and phenotypically, and peripheral populations often show a high level of inbreeding within populations (Barbaresi et al. 2003). According to the records, the red swamp crayfish was introduced into China only once (from Japan to Nanjing in

1929); therefore, it is unlikely that much gene exchange would have taken place between the Chinese and American populations (Li et al. 2005; Xia et al. 2009). The differentiation between the Taiwan and mainland populations was only moderate, mainly because the two populations are separated by the wide Taiwan Strait, and their isolation time is shorter than for the Chinese and American populations (Ibrahim et al. 1996; Li et al. 2005; Yue et al. 2010).

In this study, all the haplotype distribution rates were low, except for Hap_1, indicating that the molecular variability level was low in the Chinese population. This finding also suggests that a recent population expansion occurred within a short time period, so that there was not enough time for genetic variation to accumulate in the populations. The Taiwan population presents a variability level ($h = 0.832 \pm 0.075$, $\pi = 0.049 \pm 0.008$) that is higher than the level of variability in the mainland population ($h = 0.495 \pm 0.041$, $\pi = 0.003 \pm 0.002$). It is likely that the population structure was significantly influenced by geographic barriers (e.g., river, strait) that limit gene flow and favor interpopulation divergence, as has been found in other freshwater crustaceans (Hedgecock et al. 1979; Barbaresi et al. 2003; Yue et al. 2010). It is also likely that the genetic drift and invasion history pattern in each induced population can play important roles in the populations (Yue et al. 2010). Haplotype diversity (h) and nucleotide diversity (π) were higher in the Taiwan population than in the mainland population. Taiwan and China Mainland are separated by the Taiwan Strait, resulting in a decrease of effective gene flow between the two populations and a decline in genetic diversity in the Taiwan population. The loss of genetic variation through genetic drift and the inbreeding effect in small populations are thought to increase their extinction rate (Nichols et al. 2003; Saccheri et al. 1998). Successful invasive species such as the red swamp crayfish are generally thought to have high genetic diversity, which allows them to escape the harmful effects of inbreeding and adapt to their new environment (Keller and Waller 2002; Spielman et al. 2004).

Phylogenetic Relationship

Although geographic isolation of the populations might have contributed to their genetic differentiation, it was not the main factor in this study. The present study also showed a significant correlation between genetic differentiation and geographic distance between the Chinese and American populations. The phylogenetic analysis showed that the populations from the Yangtze and Huaihe rivers are relatively close, and they both cluster with the Yellow River population. This result indicates that the relationship between the mainland populations is close. The spread of exotic species by humans is common in freshwater ecosystems, and jump dispersal has also frequently occurred as a result of human activity (Parker et al. 1999; Suarez et al. 2001; Tiunov et al. 2006). The high dispersal ability and passive dispersal of the red swamp crayfish would have contributed to the relatively close relationship among different geographic populations. The small genetic distances between some of the populations was a bit unexpected because the populations that were sampled belong to different river systems. The results may indicate that individuals have been transferred, in the past several decades, to other provinces for culture (Yue et al. 2010).

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