



Genetic diversity among red swamp crayfish (*Procambarus clarkii*) populations in the middle and lower reaches of the Yangtze River based on AFLP markers

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ABSTRACT. The red swamp crayfish has become one of the most important freshwater aquaculture species in China. At present, although it is widely distributed in the middle and lower reaches of the Yangtze River basin, little is known about its population genetics and geographic distribution in China. We estimated the genetic diversity among 6 crayfish populations from 4 lakes (Hongze Lake, Poyang Lake, Dongting Lake, and Yue Lake) using AFLPs. A total of 129 loci were generated with 5 *EcoRI-MseI* primer combinations and scored as binary data in 139 individuals. These data were analyzed by cluster methods with the NTSYSpc software package. The 6 populations were separated into 3 major clusters by principal coordinate analysis and cluster analysis. Among the 6 populations, the highest gene diversity was found within the Nanjing population. Analysis of molecular variance demonstrated that most variation occurred within populations (91.20%). The estimated

average G_{ST} value across all loci was 0.4186, suggesting (very) low gene flow among the different localities. We conclude that there is high genetic differentiation among crayfish in the middle and lower reaches of the Yangze River. This information will help in the selection of high-quality individuals for artificial reproduction.

Key words: *Procambarus clarkii*; AFLP; Genetic diversity; Yangtze River

INTRODUCTION

The conservation of genetic diversity is important for the long-term survival of any species (Reed and Frankham, 2003). The red swamp crayfish (*Procambarus clarkii*) has moved outside of its natural ranges through various mechanisms - both natural, such as migration; accidental, such as escape from holding facilities; or deliberate, for example, through human activities (Barbaresi and Gherardi, 2000). The red swamp crayfish is the dominant benthic invertebrate when present, and may to some extent counteract the aging or overgrowth and eutrophication of small freshwater ecosystems (Skurdal and Taugbøl, 1994). This crayfish dominates the biomass of many freshwater invertebrate communities and plays a major role in food webs and community structure (Usio and Townsend, 2004). On the contrary, it also constitute the main prey of several species of fish, birds, and mammals (Rodríguez et al., 2005). In fact, the red swamp crayfish has been found to exhibit properties of an r-selected species, including early maturity at small body size, rapid growth rate (Paglianti and Gherardi, 2004), and relatively short life span (Lindqvist and Huner, 1999). Because of its aggressiveness and predatory ability, it poses threats to native fauna (Gherardi et al., 2001; Renai and Gherardi, 2004). As a successful colonizer, crayfish may reduce biomass and species richness of macro-invertebrates, macrophytes, and periphyton, including drastic habitat changes with consequent decline of vertebrate and invertebrate taxa.

Habitat loss, overfishing, and the crayfish plague have also diminished crayfish populations throughout their distribution (Oidtmann et al., 2004). It is crucial that crayfish retain as much genetic variation as possible to enhance their chances for recovery (Doyle et al., 2001).

Molecular markers are useful tools in the assessment of genetic diversity (Powell et al., 1996). Amplified fragment length polymorphism (AFLP) (Vos et al., 1995) depends on the reliability of restriction fragment length polymorphism and the high efficiency of polymerase chain reaction (PCR) to amplify digested genome DNA segments selectively. AFLP is highly reliable for the assessment of genetic variation among and within populations (Maheswaran et al., 1997). AFLP has been widely applied to study genotyping, population differentiation, and genetic diversity in a wide variety of organisms, such as catfish (Mickett et al., 2003), tilapia (Agrestia et al., 2000), and penaeid shrimp (Wang et al., 2004).

In recent years, the red swamp crayfish has become an important commercial shrimp distributed mainly in the middle and lower reaches of Yangtze River and its tributaries. However, almost nothing is known about its population genetics and invasion routes in the river. Therefore, the objectives of this study were to 1) provide reliable information for the protection and restoration of wild resources and the selection of high-quality individuals for artificial reproduction, and 2) confirm the first introduction site of the species and understand the genetic diversity and population structure and potential avenues of dispersal.

MATERIAL AND METHODS

Sample collection

From June 2009 to June 2010, we collected 139 crayfish using 15 baited cylindrical traps (4 m long with a section of 32.5 cm, 2 opposed access funnels with 10 cm in diameter, and a 0.7-cm mesh) from 6 sites in Yangtze River tributaries to study genetic diversity and dispersal. These 6 sites include Hongze Lake (HZ) in Jiangsu Province; Poyang Lake (PY^a), Ruihong Lake (PY^b), and Ruichang Lake (PY^c) in Jiangxi Province; Dongting Lake (DT) in Hunan Province, and Yue Lake (YL) in Hubei Province. The crayfish were preserved in absolute ethanol and stored at -20°C before DNA extraction.

DNA isolation and AFLP reactions

Genomic DNA was isolated from approximately 1.5 g tissue using a Universal Genomic DNA Extraction Kit Ver. 3.0 (TaKaRa Biotechnology Co. Ltd., Dalian, China) according to manufacturer instructions. We used 0.5% agarose gel electrophoresis and a spectrophotometer to test the quality of the genomic DNA. AFLP was carried out as described by Vos et al. (1995), with some modifications. Briefly, 500 ng genomic DNA from each crayfish was digested with 5 U of each *EcoRI/MseI* endonucleases and bound to their respective adapters. After incubation for 24 h at 37°C, the pre-amplification product was diluted (1:20) in ultrapure water and selectively amplified with E_{+3} and M_{+3} primers and 1.5 U DyNzyme II DNA Polymerase. The diluted sample (1 μ L) was used as a template in a 20- μ L volume of final amplification with *EcoRI/MseI* plus 3 additional nucleotide primers.

PCR amplification was carried out in a PerkinElmer 9700 thermocycler, with 2 cycles of denaturation at 94°C for 30 s, annealing at 65°C for 30 s, and extension at 72°C for 1 min, followed by 12 cycles of a touchdown phase in which the annealing temperature was lowered 1.5°C in every cycle followed by 25-28 cycles of 94°C for 45 s, 53°C for 60 s, and 72°C for 90 s. A final extension was carried out at 72°C for 5 min.

The products of selective amplification were separated on 6% polyacrylamide gels (acrylamide:bisacrylamide) for 3 h at 180 V and stained with 20% silver nitrate. Gels were fixed for 10 min with a mixed solution of 10% ethanol and 0.5% acetic acid and then stained for 30 min with 0.2 g/mL silver nitrate solution. After being washed with distilled water, the gels were developed in 1.75 g/mL sodium hydroxide solution with 0.4% formaldehyde until bands were noticeable. Finally, the gels were washed with distilled water and air dried.

Data analysis

A band was considered to be polymorphic if it was present in at least one genotype and absent in the others. A data matrix was generated in which each band was scored 1 if present and 0 if absent. The percentage of polymorphic loci, Nei's genetic diversity (Nei, 1978), Shannon index, average heterozygosity, and genetic distance (Nei, 1972) were calculated using POPGENE v. 1.31. Statistica 6.0 was used to test the difference in intrapopulation genetic distance and average heterozygosity between populations. A dendrogram of the 6 populations was constructed based on the unweighted pair-group method with arithmetic means (UPGMA) in POPGENE 3.2. Analysis of molecular variance was carried out via

Arlequin v. 3.11 (Excoffier et al., 2005) to evaluate the distribution of genetic variation within and among samples as well as to estimate the G_{ST} (coefficient of gene differentiation) index. Principal coordinate analysis (PCoA) was used to evaluate the distribution of genetic distance in clusters with the POPGENE software. The statistical significance of the total and pairwise fixation indices was estimated by comparing the observed distribution generated by 1000 permutations. Gene flow estimates were derived using the equation $N_m = 0.25 (1 - G_{ST}) / G_{ST}$.

RESULTS

AFLP and gene diversity

Selectively amplified results of 5 primer combinations were analyzed in this study. A total of 129 bands were identified in 139 individuals, and the average was 25.8 bands for each primer combination. The average heterozygosity and percentage of polymorphic loci for each population are summarized in Table 1. Great variation in genetic diversity was observed among populations, as indicated by the average heterozygosity and percentage of polymorphic loci. The greatest percent polymorphism (75.74%) was that of the HZ population; those in the YL (65.84%) and PY^b (65.12%) populations were relatively lower. A G_{ST} analysis was performed to estimate differences among locations. The estimated G_{ST} value averaged over all polymorphic loci was 0.4184, indicating a strong population structure (Table 2).

Table 1. Genetic diversity of the six population of *Procambarus clarkii*.

Population code	No. of alleles	Effective No. of alleles	Nei's gene diversity	Shannon's information index	Percentage of polymorphic loci	Sample No.
PY ^a	1.3953 ± 0.4908	1.2529 ± 0.3562	0.1471 ± 0.1959	0.2183 ± 0.2834	39.53%	24
RH (PY ^b)	1.6512 ± 0.4908	1.3931 ± 0.3138	0.2848 ± 0.1766	0.2880 ± 0.2565	65.12%	24
RC (PY ^c)	1.5349 ± 0.5007	1.2862 ± 0.3409	0.1736 ± 0.1884	0.2645 ± 0.2737	53.49%	24
HZ	1.8574 ± 0.5001	1.4181 ± 0.2080	0.5829 ± 0.1253	0.4419 ± 0.1926	75.74%	27
YL	1.7884 ± 0.5018	1.4091 ± 0.3298	0.3581 ± 0.1857	0.3412 ± 0.2710	65.84%	19
DT	1.6177 ± 0.4611	1.3807 ± 0.3496	0.2290 ± 0.1885	0.2675 ± 0.2677	63.77%	21
Mean	1.6408 ± 0.4909	1.3567 ± 0.3168	0.2959 ± 0.1767	0.3036 ± 0.2575	60.58%	23

PY^a = Poyang Lake; RH (PY^b) = Ruihong Lake; RC (PY^c) = Ruichang Lake in Jiangxi Province; HZ = Hongze Lake in Jiangsu Province; YL = Yue Lake in Hubei Province; DT = Dongting Lake in Hunan Province.

Table 2. Nei's (1988) analysis of gene diversity in *Procambarus clarkii* populations.

	Total gene diversity	Gene diversity within population	Coefficient of gene differentiation (G_{ST})	Gene flow (N_m) $N_m = 0.5 (1 - G_{ST}) / G_{ST}$
Mean	0.2797	0.1626	0.4186	0.6944
Standard deviation	0.0299	0.0096		

Population structure

The individual-based similarity trees with the highest cophenetic correlation values, and therefore the best fit between the distance matrix and corresponding tree, were produced through UPGMA cluster analysis (Figure 1). UPGMA was carried out to demonstrate graphically the genetic similarities among crayfish populations. The genetic similarity coefficient among populations ranged from a low of 0.7797 between the HZ and PY^a populations to a high of 0.9067 be-

tween the HZ and YL populations (Table 3). Nei's genetic distances for all pairwise comparisons were significantly different from zero. The genetic distance value (0.2489) between the HZ and PY populations was the highest, whereas the value (0.0979) between the YL and HZ populations was the lowest (see Table 3). Therefore, the resulting dendrogram grouped the 6 populations (100 individuals) into 3 major clusters that consisted of 2 or more accessions (see Figure 1). The first cluster consisted of 17 PY^a accessions, 6 PY^b accessions, 1 HZ accession, and 9 DT accessions. The second cluster consisted of 8 PY^b accessions, 13 HZ accessions, 15 YL accessions, and 8 DT accessions. The third cluster consisted of 18 PY^c accessions and 1 PY^b accession. Interestingly, we found that crayfish from the HZ and PY^a populations, which were geographically close and connected through the Yangtze River, could be closely clustered together. Expectedly, the PY^c population, which was located far from the others, was clustered into a separate branch.

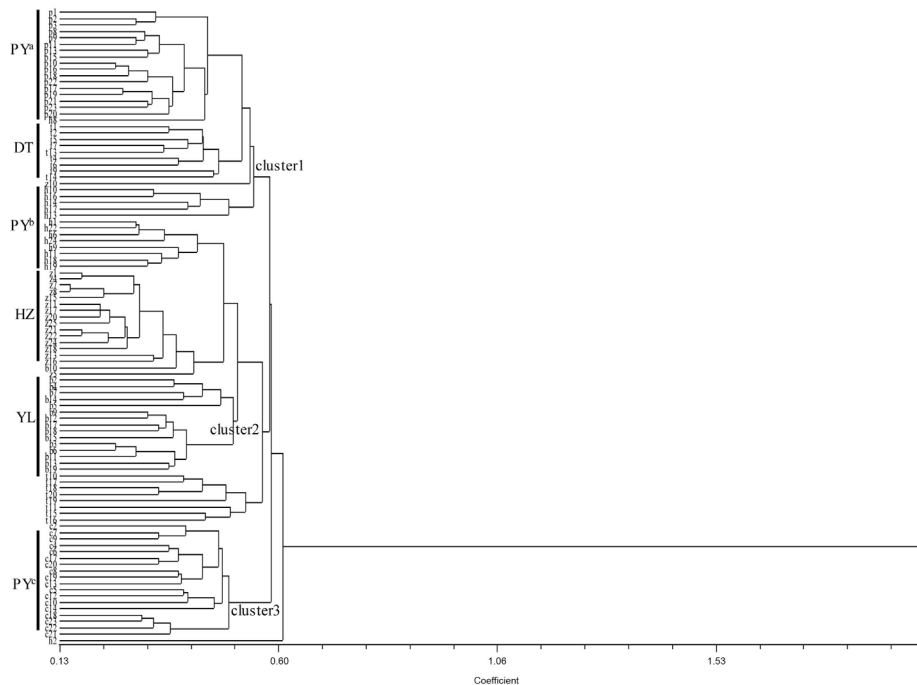


Figure 1. Principal coordinate analysis based on the distance matrix inferred from the Nei similarity index. For abbreviations, see legend to Table 1.

Table 3. Genetic similarity indices (above the diagonal) and genetic distances (below the diagonal) among 6 populations of *Procambarus clarkii*.

Population	PY ^a	RH (PY ^b)	RC (PY ^c)	HZ	YL	DT
PY ^a	-	0.8632	0.8062	0.7797	0.7904	0.8119
RH (PY ^b)	0.1474	-	0.8358	0.8745	0.8709	0.8399
RC (PY ^c)	0.2154	0.1793	-	0.8207	0.8505	0.8049
HZ	0.2489	0.1341	0.1976	-	0.9067	0.8198
YL	0.2352	0.1382	0.1620	0.0979	-	0.8743
DT	0.2084	0.1745	0.2171	0.1987	0.1343	-

For abbreviations, see legend to Table 1.

Analysis of molecular variance was conducted to describe the variance components of crayfish populations. The result revealed that 91.20% of the genetic variation was explained by variation within populations and 8.80% was explained by variation among populations (Table 4).

Table 4. Analysis of molecular variance within and among *Procambarus clarkii* populations with 5 microsatellites.

Source of variance	Sum of squared deviation	Variance component	Percentage of total variance	d.f.	P*
Variance within populations	17193.12	8.846	91.20%	133	<0.001
Variance among populations	2088.26	0.512	8.80%	5	<0.001
Total	19281.38	9.358	100.00%	138	

d.f. = degrees of freedom. *P values are the probabilities of having a more extreme variance component than the observed values alone. Probabilities were calculated by 1000 random permutations of individuals across populations.

PCoA

PCoA was also performed to display the relationship among 6 populations on 2 coordinate axes (Figure 2). Like the UPGMA clustering dendrograms, the PCoA analysis also placed 6 populations into 3 distinct groups. Up to 32.477% of the total variation was explained by the first 3 axes, which accounted, respectively, for 17.605, 9.124, and 5.748% of the observed variation. The 3 main groups from the cluster analysis appeared well separated. PY^c accessions were concentrated toward the upper part of the y-axis. HZ, YL, and PY^b accessions were concentrated toward the left part of the x-axis. Three ensembles overlapped widely, suggesting that hybridization among 3 populations may yield desirable genetic variability for further breeding and selection.

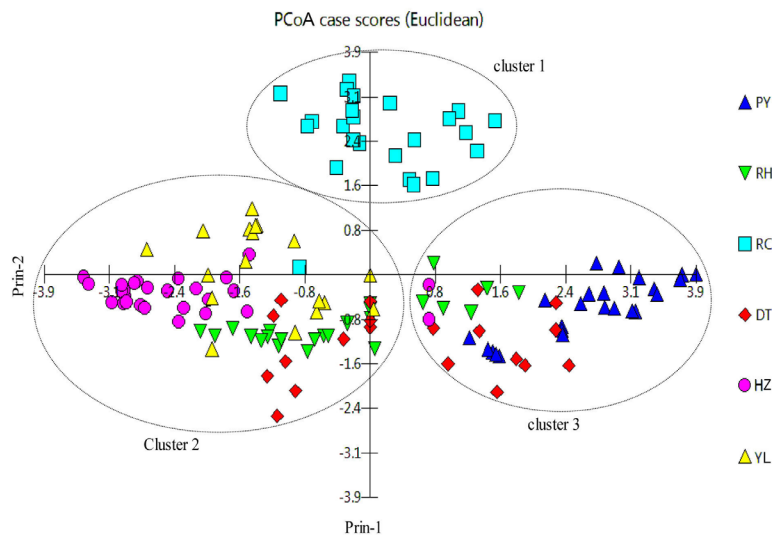


Figure 2. UPGMA dendrogram based on the Euclidean distance between the 100 sampled crayfish. PCoA = principal coordinate analysis. For population abbreviations, see legend to Table 1.

DISCUSSION

The pattern of genetic diversity observed has practical implications for the management of broodstocks and for selective breeding programs (Song et al., 2006). Genetic diversity information among and within populations of the red swamp crayfish in the Yangtze River also provides reliable information for the protection and restoration of wild resources. Our findings clearly indicated that some distinct differences exist among the 6 populations. The HZ population showed the most distinct difference with the highest allelic and gene diversity. High genetic diversity of introduced populations can be attributed to several factors: multiple introductions (Kolbe et al., 2004), a single introduction of a large number of individuals from different populations (Yue et al., 2010), and remote living locations and lower fishing pressure (Song et al., 2006). Yue et al. (2010) have proved that the population in Nanjing, Jiangsu Province, may be the first invasive population of *P. clarkii* in China and that the translocation of *P. clarkii* from locations outside of China to Nanjing did not occur. The scale of introduction and fishing intensity in the HZ population of this species was relatively lower; which may cause high genetic diversity of introduced populations.

Successful invasive species are generally thought to have high genetic diversity, which allows them to escape the harmful effects of inbreeding and adapt to their new environment (Spielman et al., 2004). However, we found that overall gene diversity varied from 0.1471 to 0.5829 in the 6 studied crayfish populations and was lower than that of populations in the United States (0.78) (Belfiore and May, 2003). Gene diversity has clearly decreased since crayfish were introduced to China in 1929 from Japan, which may have resulted from the presence of bottleneck and founder effects and nonrandom mating (Rozas et al., 1990). Yue et al. (2010) have proved that the bottleneck effect is a crucial factor in gene diversity. Founder effects have been reported in a number of invasive species, including quagga mussel (Wilson et al., 1999), zebra mussel (Astaneh et al., 2005), and Chinese mitten crab, which has invaded Europe (Herborg et al., 2007). In fact, Barbaresi et al. (2003) have shown that low genetic diversity is due to the founder effect. Because crayfish are highly fecund and can produce more than 200 eggs at each spawning (Rodríguez-Serna et al., 2000), mating between relatives is highly possible. Villanelli and Gherardi (1998) have reported that nonrandom mating and mate choice have been noted in some freshwater crayfish species. Therefore, we cannot conclude that the gene diversity within each population observed in this study was caused by a bottleneck effect and nonrandom mating.

The genetic differences among the various crayfish populations may be directly related to the distance of isolation and the connectivity level of the rivers they inhabit. The genetic distance ranged from 0.0979 to 0.2489 in our study. The genetic distance between the HZ and YL populations was the lowest, and the HZ and PY^a populations had the greatest distance owing to their far distance, in which land and large rivers might have prohibited gene flow. Hedgecock et al. (1979) have reported that the population structure of freshwater crayfish is significantly influenced by several factors: land, mountains, and water level. These factors limit gene flow and therefore favor interpopulation divergence. For example, the PY^a population exhibits a large seasonal water level fluctuation during the rainy season from April/May to October (Guo et al., 2005). If water flooded and connected all marshlands and islands, the gene interchange could occur frequently for most of the populations. However, during the dry season from November to April, up to 90% of water is lost, exposing vast grass-covered

marshlands (area: 1334 km²). Adult *P. clarkii* have difficulty swimming from their habitats to other sites. Therefore, water level is a crucial factor affecting the population structure of freshwater crayfish.

The G_{ST} value represents the degree of genetic differentiation within populations ($0 < G_{ST} < 1$). Mickett et al. (2003) have suggested that the F_{ST} (genetic differences among population) in channel catfish (0.4456) indicates a high degree of genetic differentiation, whereas Yue et al. (2004) have reported moderate genetic differentiation in Asian arowana, with a G_{ST} of 0.047. In our study, F_{ST} averaged over all polymorphic loci was 0.4186, which is similar to the result reported by Mickett et al. (2003). This value indicates a high degree of genetic differentiation among the 6 populations. Yue et al. (2010) have stated that genetic drift and selection may play important roles in genetic differentiation in *P. clarkii*. Nevertheless differences in population sizes are known to influence the degree of genetic differentiation within and between populations (Lande, 2002). Gouin et al. (2001) have maintained that human transplantation has caused genetic differentiation in the endangered freshwater crayfish. The impact of habitat variation on genetic differentiation has been illustrated in several animals, particularly crayfish (Austin and Knott, 1996). In our study, the pattern of genetic differentiation among 6 populations could be explained by 2 hypotheses. The first is based on human transplantation, which could be responsible for the differentiation. Crayfish transplantation was a common practice as early as Middle Ages, and isolated populations exist because of such activities. Thus, transplantation of crayfish from populations located in different lakes may have altered the natural genetic pattern. The second hypothesis is that this genetic pattern within and between populations can be accounted for by a period of population expansion and migration or gene flow followed by contraction, isolation, and fragmentation of populations, which leads to genetic divergence mainly under the process of genetic drift and resulted in fixation of the alternative variants in a stochastic manner. Similar patterns of genetic differentiation have been described in crayfish species using allozyme studies (Austin and Knott, 1996).

In summary, the results of genetic investigation will be useful for the development of artificial propagation and genetic improvement programs for crayfish, which give populations the ability to adapt to environmental changes and stresses. Crayfish is an extremely important economic resource in the middle and lower reaches of the Yangtze River. Furthermore, the supply of offspring from hatcheries cannot meet the demand of the market. Our research revealed that the HZ population has considerable genetic variety. Therefore, parental crayfish for artificial propagation should be collected from this population to ensure the conservation of wild resources and genetic diversity.

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