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Full Length Research Paper

Evaluation of genetic diversity in the golden apple snail, *Pomacea canaliculata* (Lamarck), from different geographical populations in China by inter simple sequence repeat (ISSR)

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The genetic diversity of *Pomacea canaliculata*, collected from Los Banos (LB) in Philippines and Yuyao (YY), Taizhou (TZ), Fuzhou (FZ), Guangzhou (GZ), Nanning (NN), Kunming (KM) in China, was studied by using the inter simple sequence repeat (ISSR) technique. A total of 498 loci from 140 individuals were amplified with four selected ISSR primers and the percentage of polymorphic loci was 87.35%. At the species level, the Nei's gene diversity (*H*) was 0.3805 and the Shannon information diversity index *(I)* was 0.5607. A relatively high level of genetic differentiation among populations was detected based on Nei's gene diversity analysis (G_{st} = 0.2001) and analysis of molecular variance (AMOVA) analysis (Φ_{st} = 0.0824), indicating the vast majority of genetic variation that occur within the populations. The limited genetic distance (0.0793) and correlation between genetic distance and geographic distance matrices (r = 0.5638, P > 0.5) indicated that, there was no significant geographic heterogeneity among these populations.

Key words: *Pomacea canaliculata*, inter simple sequence repeat (ISSR), genetic diversity, geographical populations.

INTRODUCTION

The golden apple snail (*Pomacea canaliculata,* lamarck), indigenous to South America, was introduced from Argentina to Taiwan in the 1980s for commercial production (Mochida, 1991) and was distributed widely in Asia as a dietary protein supplement and income earner for the rural poor. However, the introduction was done in haste with no prior studies on market information and the ecological impact (Halwart, 1994; Naylor, 1996). Golden apple snails damage a wide range of plants such as algae, azolla, duck weed, water hyacinth, rice seedlings and other succulent leafy plants. In China, the yield loss caused by *P. canaliculata* was from 10 to 90% in rice field and from 10 to 20% in *Zizania caduciflora* field (Yu et al., 2001).

Growth and reproduction of *P. canaliculata* were not the same from different regions. The review by Cowie (2002) indicated that, *P. canaliculata* in the Philippines behaved in a similar way with the snails in Sabah, but in Japan and Argentina, the snails took a longer time to reach maturity and hatching success being lower with fewer eggs in each cluster. In Argentina, it took between 7 months to 2 years for snails to mature (Estebenet and Martin, 2002). In Hawaii, a sex ratio of 1:1 and a maximum shell size of only 30 mm were reported by Cowie (2002), whereas in Thai- land the shells can reach at least 65 mm in height (Keawjam and Upatham, 1990). Therefore, allopatri

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Abbreviations: AMOVA, Analysis of molecular variance; **ISSR,** inter-simple sequence repeats; **PCR,** polymerase chain reaction; **PPBs,** percentage of polymorphic bands; **RAPD,** randomly amplified polymorphic DNA; **RFLP,** restriction fragment length polymorphism.

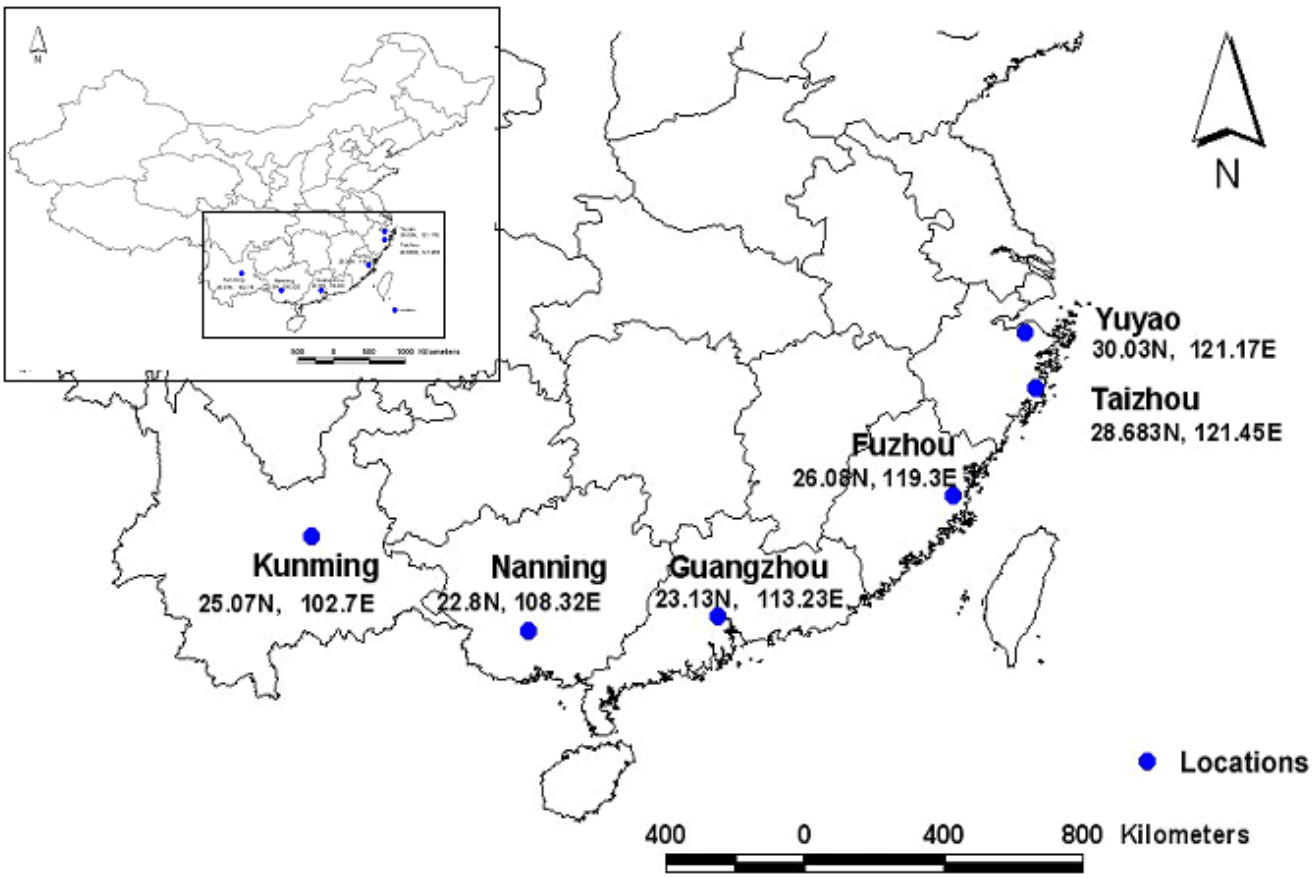

Figure 1. Map showing locations and population of *P. canaliculata* sampled.

populations inhabiting different habitats may show eco-morphological variations and a questionable species status. It was suggested that the apple snails had a high adaptability to the new environment and was easier to form a new populations.

In China, *P. canaliculata* was first introduced to Guangzhou in 1981, and now it spreads throughout the southeast and southwest of China, including Guangdong, Guangxi, Hainan, Taiwan, Fujian, Yunnan, Sichuan, Zhejiang, Jiangxi and Jiangsu provinces (Yu et al., 2001). The golden apple snails in China exhibited variability in shell color and banding pattern, which ranged from golden yellow to olive yellow and dark olive green, with numbers of small and large bands. In our previous study, we found *P. canaliculata* from different provinces in China, showed different resistances to high and low temperatures (Pan et al., 2008), but the conservative sequences (such as 16S rRNA gene, 18S rRNA gene, 28S rRNA gene and COI gene) from these populations were not diverged (Pan et al. 2009). These results indicated that, genetic structure was not altered. In order to elaborate genetic diversity from these populations in China, a search for molecular mar- kers is required to precisely quantify the genetic diversity and to molecularly characterize these species. In this study, we developed the inter-simple sequence repeats (ISSR), which was believed to be a powerful technique to assess genetic diversity among closely related species and to detect similarities between and within population levels (Moreno et al., 1998; Ghariani et al., 2003). Due to this, the levels of genetic diversity of the introduced *P. canaliculata* in China were evaluated based on ISSR data.

MATERIALS AND METHODS

Sampling and DNA extraction

The golden apple snail were collected from Yuyao (YY) and Taizhou (TZ) in Zhejiang province, Fuzhou (FZ) in Fujian province, Guang-zhou (GZ) in Guangdong province, Nanning (NN) in Guangxi province, Kunming (KM) in Yunnan province in China (Figure 1) and one population from Los Banos (LB) in the Philippines. The tested *P. canaliculata* was identified with the conserved sequence reported by Matsukura et al. (2008) and Pan et al. (2009). A total of 140 individuals of snails, based on seven sample sets with 20 individuals each, were collected in the present study. Specimens were then frozen at -70°C or stored in 70 - 100% ethanol. Ethanol-preserved samples were re-hydrated in sterile double H_2O for at least 2 h before extraction. The total DNA of each snail was extracted from ~50 mg of tissue using standard phenol/chloroform methods or Qiagen's Dneasy extraction kit. DNA concentration was determined spectro- photometrically and extracted DNA was diluted to 30～50

Table 1. Attributes of primers used for generating ISSR amplification and number of bands per primer.

Primer	Sequence 5'→3'	Annealing temperature (°C)	No. of bands scored	No. of polymorphic bands	Percentage of polymorphic bands (%)
UBC808	$(AG)_8C$	50	123	111	90.24
UBC811	$(GA)_8C$	52	123	102	82.93
UBC868	$(GAA)_6$	48	144	126	87.5
UBC901	$(CA)_8RY$	46	108	96	88.89
Total			498	435	87.35
Mean			124.5	108.8	

ng/µl in ddH_2O and stored at -20°C until required.

ISSR–polymerase chain reaction (PCR) amplification

For ISSR–PCR analysis, a total of 90 primers from UBC (University of British Columbia) primer set (http://www.biotech.ubc.ca/services/naps/primers) were screened and four primers which produced clearly reproducible bands were selected for ISSR analysis (Table 1). The effects of Mg^{2+}, dNTPs, DNA templates, primers and DNA polymerase on the amplification were tested and the optimal reaction system of ISSR for *P. canaliculata* was determined. PCR reactions were carried out on a thermocycler and programmed for pre-denaturing at 94°C for 3 min, followed by 26 cycles of 94°C for 30 s, 46, 48, 50 or 52°C for 45 s (Table 1), 72°C for 1 min and 7 min at 72°C for final extension. Reactions were carried out in a volume of 20 µl, containing 0.2 mM of each dNTP, 1.5 mM $MgCl_2$, 0.5 µM primer, 1 U Taq polymerase and 10 ng DNA template. Amplification products were separated by electrophoresis on 6% polyacrylamide gels, visualized by silver staining and photographed (Li et al., 2009). Sizes of amplification products were estimated using DNA marker DL2000. Negative controls and lacking template DNA, were included in each PCR set to test for the possibility of contamination.

Data analysis

Amplified products were scored as presence (1) or absence (0) for each DNA sample to form a binary matrix. Fragments that could not be scored unambiguously were not included in the analysis. The resulting binary data matrix was analyzed using POPGENE Version 1.32 (Yeh et al., 1997). Genetic diversity within and among populations was measured by the percentage of polymorphic bands (PPBs), Nei's gene diversity (*H*) and Shannon's information index (*I*). Genetic differentiation among populations was estimated by gene differentiation coefficient (G_{st}) (Nei, 1973). Corresponding estimates of gene flow (N_m), that is, the average per generation number of migrants exchanged among populations, was related to G_{st} according to the equation $N_m = (1/G_{st} - 1)/4$. All these calculations assumed that populations were in Hardy–Weinberg equilibrium, in which cases of allele frequencies were estimated based on the square root of the frequency of the null (recessive) allele. The measurement for partitioning genetic variation was obtained with the hierarchical AMOVA analysis, using WINAMOVA 1.55 (Excoffier et al., 1992). Variance components, which is the sum of all squared differences and analogues of *F*-statistics (Φ) based on Euclidean distance between individuals was calculated to estimate the population differentiation.

An unweighted pair group method with the arithmetic averaging (UPGMA) dendrogram was constructed based on the matrix of Nei's unbiased genetic distances and 1000 times bootstrapping using program TFPGA (Miller, 1997). To test the correlation between genetic distances and geographical distances (in km) among populations, a Mantel test was performed using the program NTSYS-pc 2.10e with 3000 permutations.

RESULTS AND DISCUSSION

Genetic diversity

Four selected ISSR primers generated 498 bands ranging from 150 to 2500 bp (Figure 2), corresponding to an average of 124.5 bands per primer (Table 1). Of these bands, 87.35% (435 in total) were polymorphic among 140 individuals, that is, the percentage of polymorphic bands for this species was 87.35% and the total number of bands and polymorphic bands produced by each primer were different. The highest number of bands was produced by UBC 868, but the highest level of polymorphic bands was produced by UBC 808 (Table 1).

Nei's gene diversity (*H*) varied between 0.2612 and 0.3340, with an average of 0.3044, and arranged in a decent order of LB > KM > NN > FZ > TZ > GZ > YY populations. The Shannon's information index (*I*) ranged from 0.3910 to 0.4856, with an average of 0.4499. The values of *H* and *I* showed a similar trend to PPB (Table 2); when calculated at the species level, the *H* and *I* values equaled 0.3805 and 0.5607, respectively. Among the 7 populations investigated, the highest and lowest levels of genetic variability occurred in population LB (PPB: 84.34%; *H*: 0.3340; *I*: 0.4856) and population KM (PPB: 77.10%; *H*: 0.2612; *I*: 0.3910), respectively.

The total gene diversity (H_t) was 0.3805 ± 0.0136, and the gene diversity within populations (H_s) was 0.3044 ± 0.01 while the gene diversity between populations (G_{st}) was 0.2001. 79.99% of genetic variation existed within populations. The limited gene flow (N_m = 0.9993) exist among populations.

Genetic relationships

According to AMOVA analysis, there were highly significant ($P < 0.001$) genetic differences among the seven populations of *P. canaliculata*. Of the total genetic

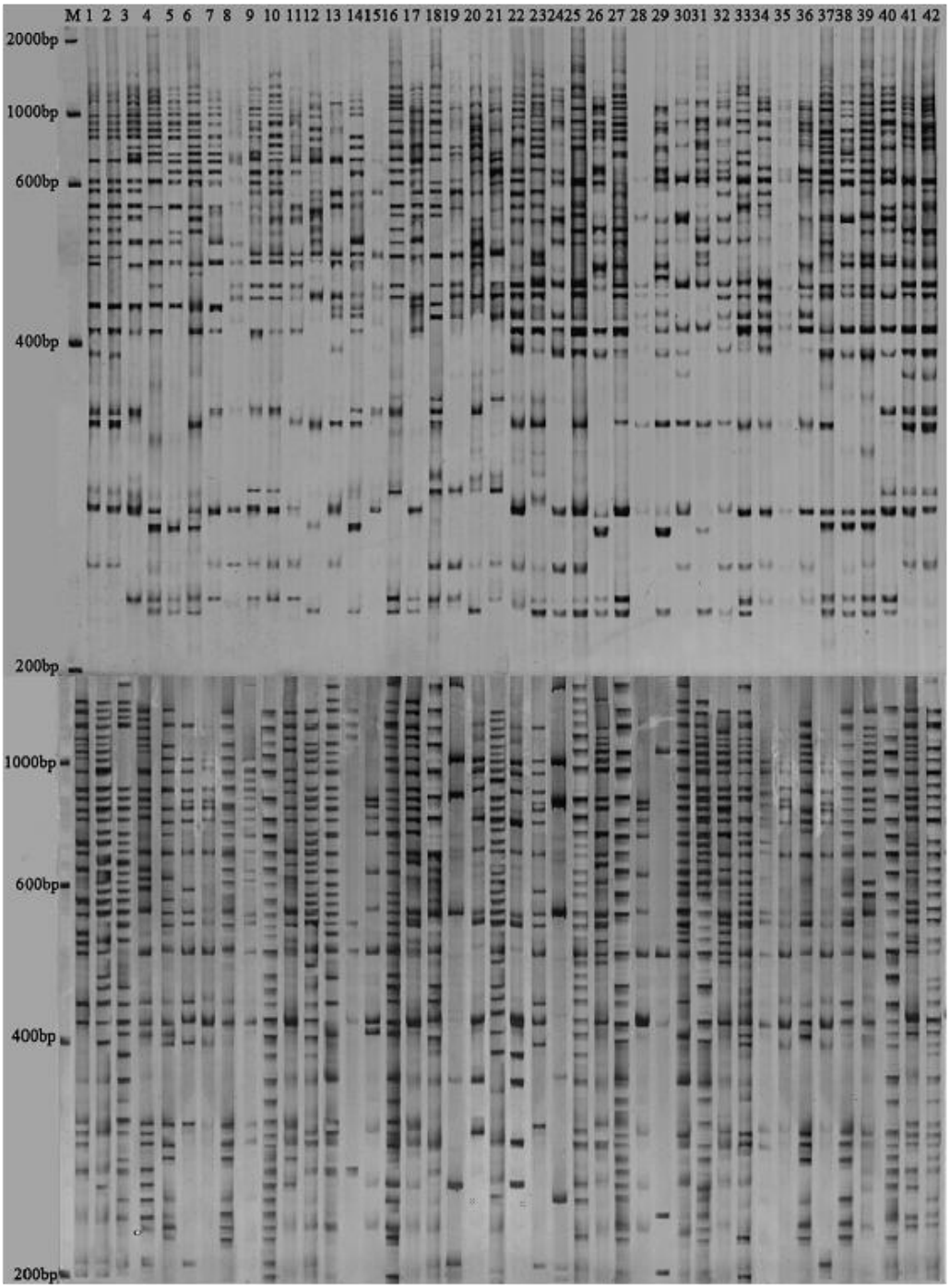

Figure 2. The amplification patterns with primers ISSR-808 (upper) and ISSR-868 (below) in seven populations of *P. canaliculata.* The lanes from left to right were DL 2000 DNA Marker (M); Population of Taizhou (1 to 6), Yuyao (7 to 12), Fuzhou (13 to18), Guangzhou (19 to 24), Nanning (25 to 30), Kunming (31 to 36) and Los Banos (37 to 42).

diversity, 8.24% was attributed to among populations and the rest (91.76%) to differences within populations (Table 3). Thus, AMOVA (Φ_{st} = 0.0824) also supported the results of Nei's gene diversity statistics and Shannon's information measure that there was a relatively high level of genetic differentiation among populations.

Estimates of genetic distances (F_{st} based values) between pairs of populations were calculated based on 166 markers scored. Values ranged from 0.0204 (between KM and NN) to 0.1780 (between TZ and LB), with

Table 2. Genetic diversity within populations of *P. canaliculata.*

Population	Percentage of polymorphic bands (PPB, %)	Nei's gene diversity (*H*)	Shannon's information index (*I*)
TZ	81.33	0.2983 ± 0.1806	0.4437 ± 0.2475
YY	74.10	0.2612 ± 0.1914	0.3910 ± 0.2668
FZ	81.93	0.3090 ± 0.1748	0.4593 ± 0.2378
GZ	80.72	0.2890 ± 0.1779	0.4323 ± 0.2462
NN	82.53	0.3142 ± 0.1812	0.4629 ± 0.2480
KM	82.53	0.3250 ± 0.1832	0.4744 ± 0.2531
LB	84.34	0.3340 ± 0.1835	0.4856 ± 0.2519
Mean	81.07	0.3044 ± 0.1818	0.4499 ± 0.2502
Species level	87.35	0.3805 ± 0.1165	0.5607 ± 0.1382

Table 3. Genetic distance (FST-based values; below diagonal) and P-value (above diagonal) among seven populations of *P. canaliculata* based on ISSR data.

Source of variation	d.f.	Variance component	Total variance (%)	Fixation index	P
Among populations	6	2.90	8.24	Φ_{st} = 0.082	□0.001
Within populations	133	32.25	91.76		
Among regions	2	1.27	3.57	Φ_{ct} = 0.036	□0.001
Among populations/regions	4	1.99	5.6	Φ_{sc} = 0.058	□0.001
Within populations	133	32.35	90.83	Φ_{st} = 0.092	□0.001

Analysis of molecular variance (AMOVA) for 140 individuals of *P. canaliculata* using ISSR markers. Two AMOVAs including nested analysis (between regions, among populations within regions and within populations) and among population analysis (among populations and within) were used. Significance tests after 9999 random permutations.

Table 4. Genetic distance (F_{st}-based values; below diagonal) and *P*-value (above diagonal) among seven populations of *P. canaliculata* based on ISSR data.

Population	TZ	YY	FZ	GZ	NN	KM	LB
TZ		0.4890	0.3180	0.2467	0.0980	<0.001	<0.001
YY	0.0265		0.3678	0.1458	<0.001	0.0875	0.1384
FZ	0.032	0.0419		0.3872	0.1793	0.2783	0.0492
GZ	0.0877	0.0975	0.0269		0.3290	0.2730	0.1844
NN	0.0907	0.1269	0.0627	0.0400		0.5620	0.1953
KM	0.0757	0.1028	0.0432	0.0643	0.0204		0.0862
LB	0.1473	0.1780	0.1244	0.1013	0.0937	0.0816	

a mean of 0.0793 (Table 4). The pairwise F_{st} based distance values in Table 4 also indicated that no significant hete- rogeneity occurred among the seven samples except between TZ and KM, between TZ and LB, and between NN and YY. To further reveal the relationships among populations, cluster analysis (UPGMA) was used to gene- rate a dendrogram based on Nei's genetic distances (Figure 3). Seven populations of *P. canaliculata* were clustered into three groups: Group 1 or region 1 comprised of three populations (population YY, TZ and FZ) collected from locations with higher latitudes, while populations NN, KM and GZ collected from lower latitudes formed Group 2 or region 2. Population LB, which showed relatively distant genetic relationship with the others, formed an independent cluster (region 3). The AMOVA of the proportion of diversity among three regions indicated that a small amount of variation (3.56%) occurred among the three regions, with 5.6 and 90.83% between populations within regions and within a population, respectively (Table 3). Mantel test revealed no significant correlation between genetic distance and geographic distance matrices (r = 0.5638, P > 0.5).

Genetic polymorphism may be an indicator of the inte-

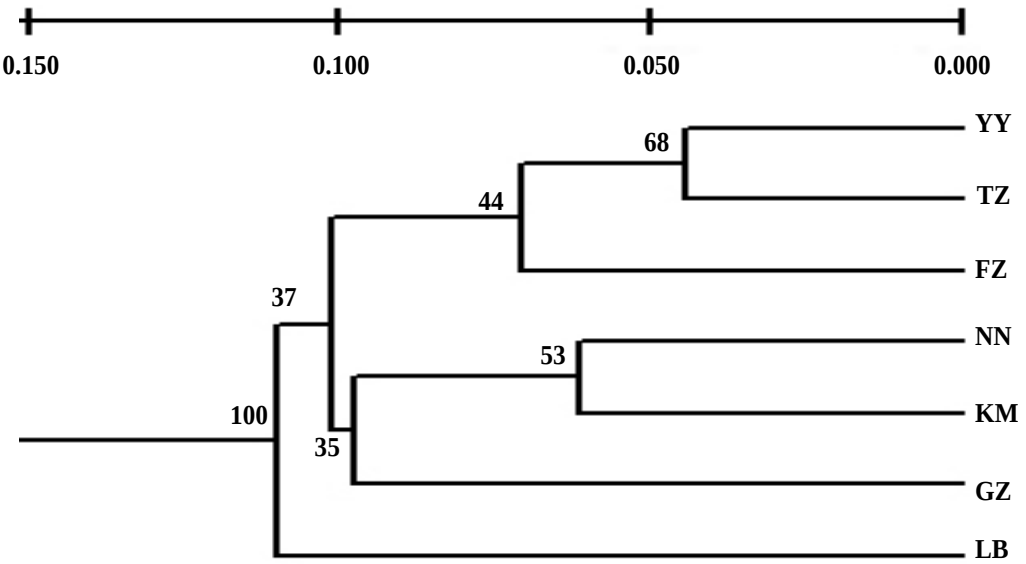

Figure 3. Dendrogram for seven populations of *P. canaliculata* using UPGMA. The numbers marked on the branches are bootstrap values (%) out of 1000 bootstraping.

ractions of various processes, such as the long-term evolutionary history of the species (for example, shifts in distribution, habitat fragmentation, and population isolation), mutation, gene flow, genetic drift and selection (Schaal et al., 1998). The snails showed a high adapt- ability to the new environment and were easier to form a different genotype (Caldeira et al., 2001). To analyse the environment on the population differentiation of snails, several molecular marker methods were reported, such as randomly amplified polymorphic DNA (RAPD) (Thaewnon- ngiw et al., 2003), single stranded repeats (SSR)-PCR (Mavárez et al., 2000), polymerase chain reaction amplification and restriction fragment length polymorphism (RFLP) (Caldeira et al., 1998). In this study, ISSRs were also proved to be a high-resolution method useful for the detection of variation among or within populations of *P*. *canaliculata*. Regardless of the relatively small sample sizes, 435 polymorphic ISSR loci were sufficient to detect variation and to differentiate *P*. *canaliculata* populations with different geographical origins and should be useful for the assessment of genetic structures of golden apple snails. Under the assumption that each band represents a genetic locus, it was possible to estimate allelic frequencies and therefore to proceed further in estimating classical population parameters.

This study revealed a relatively high level of genetic diversity in *P*. *canaliculata* based on ISSR markers. At the species level, a high PPB value was detected in this species (PPB = 87.35%). Compared with the low level of inter-population genetic variability, relatively high genetic diversity occurred within populations. At the population level, the PPB values ranged from 74.10 to 84.34%, displaying a similar trend to the parameters *H* and *I*. Besides, Nei's analysis of gene diversity and AMOVA analysis revealed that the vast majority of genetic variation occurred within populations (G_{st} = 0.2001, Φ_{st} = 0.0824). The high genetic diversity in the present study was more similar to the RAPD analysis in the study of Thaewnon-ngiw et al. (2003), which represent 98.86% polymorphic bands diversity. The high genetic polymorphism in *P*. *canaliculata* may be as a result of multiple introductions to China from different regions (Hays et al., 2008), which also occurred in Japan, where at least two groups of *P*. *canaliculata* was introduced (Matsukura et al., 2008). However, our results are inconsistent with another study by Thaewnon-ngiw et al. (2004), who presumed the nucleotide diversity was relatively low in *P*. *canaliculata* based on COI polymorphism. It also resulted to the esti- mation of nucleotide diversity based solely on the band of RFLP patterns and the digestion of COI with 3 restriction enzymes not sufficient for estimates of genetic diversity in that study (Thaewnon-ngiw et al., 2004). In this study, a relatively high gene flow for *P*. *canaliculata* (N_m = 1.9986) was estimated and higher than one successful migrant per generation, indicating considerable gene flow occurring among populations. This may be due to the introduction of *P*. *canaliculata* to China only about 38 years or so, about two generations a year and the occurrence of *P*. *canaliculata* in China may be about 76 generations. In addition, the young apple snails and egg masses can be attached to the soil or the roots and of stem base aquatic plants and spread with transportation. The developed water network in southern China was also conducive to the spread of apple snails. The limited genetic distance (0.0793) also indicated that there was no geographic heterogeneity among populations, which was also supported by the mantel test, showing no significant

correlation between the geographical distance matrix and the pair- wise distance matrix.

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