# Microsatellite analysis of genetic diversity and population structure of freshwater mussel (*Lamprotula leai*)

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## ABSTRACT

*Lamprotula leai* is one of the most commercially important freshwater pearl mussels in China, but there is limited data on its genetic diversity and population structure. In the present study, 119 individuals from four major geographical populations were investigated using 15 microsatellite loci identified via cross-species amplification. A total of 114 alleles were detected, with an average of 7.6 alleles per locus (range: 2 to 21). Among the four stocks, those from Hung-tse Lake and Poyang Lake had the lowest (0.412) and highest (0.455) observed heterozygosity respectively. The polymorphism information content (*PIC*) ranged from 0.374 to 0.927 (mean: 0.907). AMOVA showed that 12.56% and 44.68% genetic variances were among populations and within individuals, respectively. Pairwise *Fst* ranged from 0.073 to 0.146, indicating medium genetic differentiation among the populations. In aggregate, our results suggest that inbreeding is a crucial factor accounting for deviations from Hardy–Weinberg equilibrium at 12 loci. Moreover, the genetic distance among four stocks ranged from 0.192 to 0.890. Poyang Lake and Hung-tse Lake were clustered together, joined with Dongting Lake and Anqing Lake. Given that specimens from Hung-tse Lake showed the highest average allele richness, expected heterozygosity and *PIC*, this location may be the source of the highest quality germplasm resources and the stock from this area may be the best for future breeding efforts.

**Keywords:** *Lamprotula leai*; Freshwater mussel; Genetic diversity; Population structure; Microsatellite loci

## INTRODUCTION

Genetic diversity is important for sustainable exploitation of cultured resources (Afanas'ev et al, 2006; Laikre et al, 2005), especially as the exploitation of aquatic stocks and environmental degradation of habitat becomes more commonplace. Higher levels of genetic diversity among these stocks often grants them greater ability to respond to environmental changes, artificial selection and pathogen infection, all of which tend to occur in during intensified aquaculture (Liu & Yao, 2013; Wu et al, 2013). For example, *Lamprotula leai* is an endemic species distributed in large and medium rivers and lakes across China (Hu, 2005) that are widely used in pearl aquaculture and indigenous handicrafts due to their large shell, strong ability to secrete pearls, and thick nacre (Wang et al, 2007). Likewise, this mussel is widely consumed as food throughout China (Liu et al, 1979). Despite the importance of this species, little is known about the current state of its genetic diversity, except that among cultured species there is germplasm degradation and general declines genetic diversity, potentially due to intensive farming and the method of cultivation (Ling, 2005). Due to the intensive cultivation of this species, it is now listed as a first-class protected aquatic wildlife species in Anhui province and second-class protected species in Hubei province (Xu et al, 2012).

To date, most of the research into *L. leai* has focused on age and growth (Ling et al, 2005), conservation biology (Ling, 2005), embryonic development (Zhang et al, 2009), abnormal development and effective accumulated temperature of parasitic glochidium (Zhang et al, 2010a), and growth and development of juvenile mussels (Zhang et al, 2010b). However, little work has been done on this species' genetic diversity and population structure. One strategy to improve our understanding of population structure and genetic diversity of many aquaculture species is molecular analysis (Liu & Corde, 2004). Microsatellite markers have been shown to be suitable tools to assess genetic diversity because of their intrinsic genetic characteristics, including high

polymorphism, stability and specificity, and co-dominant inheritance (Sun et al, 2008). More promisingly for studying *L. leai,* microsatellites have been previously used to analyze genetic diversity in several mollusks, including *Hyriopsis cumingii* (Ji, 2007), *Cristaria plicata* (Ji et al, 2007), *Pinctada martensii* (Yan et al, 2009), and *Anodonta woodiana* (Wang et al, 2011a). To date, 18 pairs of microsatellite primers have been isolated using magnetic bead hybridization and 5'-anchored PCR methods (Xu et al, 2011, 2012). In this study, we used these microsatellites to analyze the genetic relationships among four different stocks of *L. leai* and provide a novel theoretical basis for genetic resource protection and genetic management.

## MATERIALS AND METHODS

### Sample collection and DNA isolation
Freshwater mussel specimens originating from four geographical locations across China—Poyang Lake (PY), Dongting Lake (DT), Hung-tse Lake (HZ), and Anqing Lake (AQ)—were obtained from the Weiwang Pearl Cultivation Base in Zhejiang Province (Figure 1), with 31, 31, 28, and 29 samples respectively from PY, DT, HZ and AQ (Hale et al, 2012). Genomic DNA was extracted from muscular tissue following a standard phenol: chloroform protocol as published previously (Sambrook & Russell, 2001).
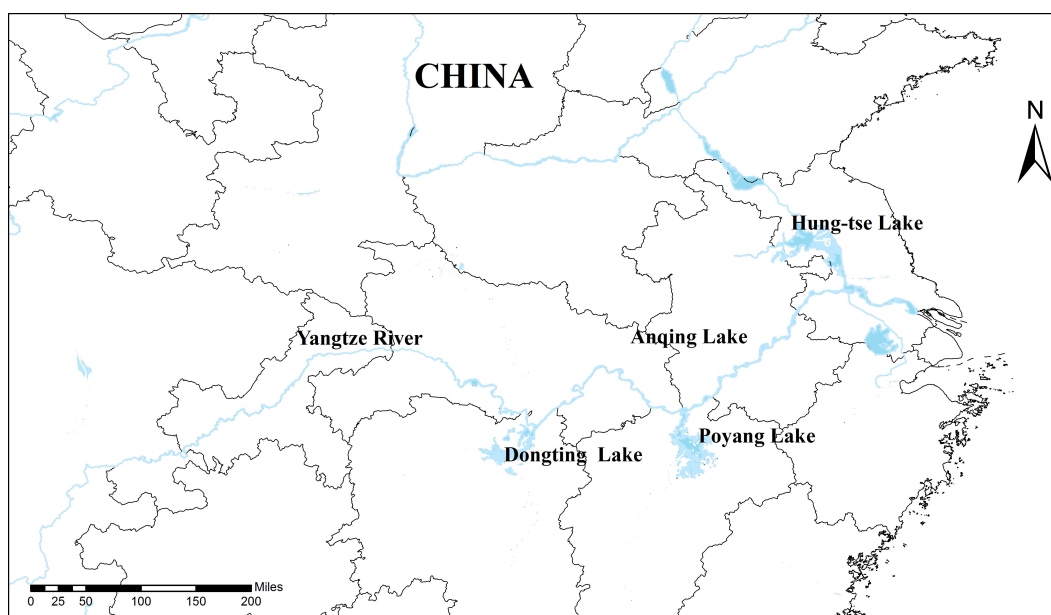


**Figure 1   Sampling locations of four *Lamprotula leai* stocks in China**

### Microsatellite amplification in *L. leai*
We used microsatellite primers published for *H. cumingii* (Bai et al, 2009; Li et al, 2007; Luo, 2006; Wang et al, 2006; Xu et al, 2010; Zhu et al, 2010) and other species closely related to *L. leai* (Ji, 2007; Launey & Hedgecock, 2001). A total of 107 candidate primer pairs (see supplemental Table 1, supporting information of http://www.zoores.ac.cn/) were synthesized (Shanghai Sangon Company) and each microsatellite was amplified in a 25 µL PCR containing 50 ng of DNA, 1 µL each of 10 µmol/L primer, 2.5 µL of 10× buffer, 2 µL of dNTP (10 mmol/L), 1 U of Taq polymerase (5 U/µL), and 17.3 µL of ddH$_2$O. PCR was conducted under the following conditions: 4 min denaturation at 94 °C 32 cycles of 30 s at 94 °C, 30 s at specific annealing temperatures, and 30 s at 72 °C; and a final extension at 72 °C for 10 min. PCR products were electrophoresed on 2% agarose gel, using 0.5% TBE buffer. Fragment sizes were determined by gel imaging analysis based on DNA Marker (ΦX174-Hinc II digest). Finally genotypes were exported to Excel tables for data analysis (An et al, 2012).

### Data analysis
The allele number ($N_A$) and observed ($H_O$) and expected ($H_E$) heterozygosity were analyzed using Popgene 1.32 (Yeh et al, 1999). Allele richness ($A_R$) was calculated using FSTAT 2.9.3 (Hered, 1995). Since allele number is influenced by sample size, we used allele richness for comparison (Yan & Zhang, 2004). Deviations from Hardy-Weinberg equilibrium (HWE) and linkage disequilibrium were estimated using Genepop 4.2 (Rousset, 2008). Meanwhile the Bonferroni correction was conducted using SPSS 18.0. The presence of null alleles was detected using Micro-checker 2.2.3 (Van Oosterhout et al, 2004). Polymorphism information content (*PIC*) was then confirmed using Microsatellite Toolkit (Zhang et al, 2010c).

The *F*-statistics (*Fis*, *Fst*, and *Fit*) and gene flow ($N_m$) were calculated by Genetix 4.05. AMOVA was conducted using Arlequin 3.1 to estimate genetic variation within and between populations as well as for individuals (Excoffier et al, 2005). Popgene 1.32 was used to calculate Nei's unbiased genetic distance between populations (Nei, 1978). A UPGMA system evolutionary tree was constructed using MEGA 5.05.

## RESULTS

A total of 15 polymorphic microsatellite loci were detected from 107 candidate primer pairs in the four tested stocks of *L. leai* via cross-species amplification. The values of $N_A$, $A_R$, $H_O$, $H_E$, *PIC* and *P* for testing HWE ($P_{H-W}$) at each locus in each stock are presented in Table 1. Totally, 114 alleles were detected. The allele number at each locus ranged from 2 to 21 (mean: 7.6). Overall, specimens from HZ showed the highest average allele richness (7.743). *PIC* values for the four stocks ranged from 0.374 to 0.927 (mean: 0.907). Collectively, stocks from DT (0.412) and AQ (0.455) had the lowest and highest *Ho*, respectively, while AQ had the lowest $H_E$ (0.791) whereas HZ had a relatively high $H_E$ value (0.868). When the four stocks were treated as one population, no significant linkage disequilibrium among the loci was detected ($P>0.05$), though 12 loci showed significant ($P<0.05$) or highly significant ($P<0.01$) deviations from HWE (Table 1).

F-statistics for 15 microsatellite loci among all four *L. leai* stocks placed the mean values of *Fis*, *Fst* and *Fit* at 0.462, 0.523 and 0.114, respectively. Pairwise comparisons revealed that the *Fst* ranged from 0.073 to 0.146 ($0.05<Fst<0.15$), indicating a medium differentiation among the four populations that was moderately closer to no differentiation (Wright, 1965). The number of migrats per generation (*Nm*) ranged from 1.46 to 3.18 (Table 2).

AMOVA analysis showed that most (44.68%) of the genetic variation originated within individuals with only 12.56% variation between the four tested populations (Table 3). The genetic distance matrix data indicated that PY and HZ populations had the smallest genetic distance (0.192) and the highest genetic similarity (0.563). Likewise, populations from DT and PY showed the greatest genetic distance (0.890) and lowest genetic similarity (0.287) (Table 4). The UPGMA dendrogram further showed that PY and HZ grouped together and then gathered with DT and AQ, which clustered together (Figure 2).
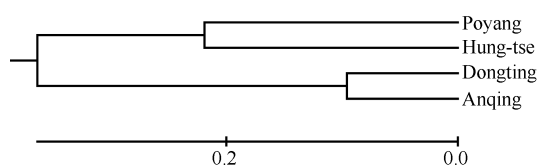


**Figure 2  UPGMA dendrogram based on Nei's genetic distances among *Lamprotula leai* stocks**

## DISCUSSION

### Genetic diversity within populations
Higher levels of genetic diversity among intensively cultivated stocks of aquatic species typically grants enhanced evolutionary potential for dealing with enviromental change, artificial selection, and pathogen infection (Liu & Yao, 2013; Wu et al, 2013), thereby improving yields and minimizing economic losses associated with these factors. In the present study, we wanted to provide basic data on the genetic diversity and stock structure of *L. leai* using microsatellite markers to provide a foundaion for a more comprehensive genetic resource protection and genetic management. Previous studies found that sample size do not always correlate with expected heterozygosity, which can be a comparison parameter (Maudet et al, 2002; Sun, 1996). Compared with other mollusks, *L. leai* showed a higher than expected heterozygosity. Previously, Li et al (2009) used eight microsatellite markers to investigate the genetic status of *H. cumingii*, with an expected heterozygosity of *H. cumingii* in PY at 0.706, which was less than the value for *L. leai* (0.835) that we arrived at in this study. Shen et al. (2013) had previously isolated 14 polymorphic loci for *Mytilus coruscus* with an expected heterozygosity averaged at 0.82, while Xu et al (2011) estimated the mean expected heterozygosity 0.683 and 0.759 using magnetic bead hybridization and 5'-anchored PCR methods to isolate microsatellite markers of *L. leai*. The disparity in these disparate findings suggests that cross-amplification may be more effective as compared with other methods. This supposition is supported by our present results, we showed that the overall level of heterozygosity was high and the *PIC* of 15 loci in this study showed high polymorphism (*PIC*>0.5). Together, these findings indicated that the stocks of *L. leai* have abundant genetic diversity (Botstein et al, 1980), but also that microsatellite loci can be used to analyze the genetic diversity and structure of mollusks like *L. leai*. More specifically, our findings showed that specimens from Hung-tse Lake had the highest $A_R$, average $H_E$ and *PIC*, suggesting specimens from this location have a potentially larger amount of genetic resources, making them ideal candidate for future breeding efforts in China.

### Genetic differentiation among populations
Genetic differentiation is commonly measured by *Fst*, *Nm* and genetic distance. Pairwise comparisons revealed medium genetic differentiation among the populations. AMOVA analysis also showed a 12.56% variation existed among these stocks (Wang et al, 2011b). Genetic differentiation can be caused by several different factors, including migration, genetic drift and gene mutation. Since the *Nm* values found in this study were all greater than 1, it is likely that genetic drift is not the main factor among *L. leai* (Slatkin, 1985). However, it seems more likely that this phenomenon may have been caused by short-time farming, as intensive farming could affect the variation among stocks.

The UPGMA cluster indicated that DT and AQ clustered together. One potential reason for this patter is that individuals of *L. leai* in Anqing Lake may have initially came from Dongting Lake. This possibility caused by either manual intervention or species migration. Study showed that genetic communication may related to geographic location (Ma et al, 2007). Here Poyang Lake in the middle and lower reaches of the Yangtze River clustered with Hung-tse Lake, which is in the downstream of Huaihe River (Xu et al, 2013). These two lakes in close geographic proximity had similar genetic relationships. However, this conclusion remains to be more verification.

**Table 1  Allele number ($N_A$), allele richness ($A_R$), observed heterozygosity ($H_O$) and expected heterozygosity ($H_E$), polymorphism information content ($PIC$), and $P$ value for testing Hardy–Weinberg equilibrium ($P_{H-W}$) in four _Lamprotula leai_ stocks**

| GenBank accession no./locus | PY(n=28) | | | | | | DT(n=31) | | | | | | HZ(n=29) | | | | | | AQ(n=31) | | | | | | All stocks | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | $N_A$ | $A_R$ | $H_O$ | $H_E$ | $PIC$ | $P_{H-W}$ | $N_A$ | $A_R$ | $H_O$ | $H_E$ | $PIC$ | $P_{H-W}$ | $N_A$ | $A_R$ | $H_O$ | $H_E$ | $PIC$ | $P_{H-W}$ | $N_A$ | $A_R$ | $H_O$ | $H_E$ | $PIC$ | $P_{H-W}$ | $N_A$ | $A_R$ | $H_O$ | $H_E$ | $PIC$ | $P_{H-W}$ |
| GQ302635 | 12 | 9.87 | 0.679 | 0.922 | 0.898 | 0.367 | 11 | 10.98 | 0.355 | 0.889 | 0.863 | 0.000 | 12 | 10.96 | 0.483 | 0.917 | 0.893 | 0.006 | 10 | 9.56 | 0.452 | 0.849 | 0.815 | 0.004 | 12 | 10.75 | 0.487 | 0.966 | 0.961 | 0.000 |
| GQ302636 | 5 | 4.90 | 0.357 | 0.779 | 0.726 | 0.000 | 7 | 6.99 | 0.226 | 0.812 | 0.769 | 0.000 | 5 | 4.00 | 0.241 | 0.763 | 0.710 | 0.002 | 7 | 6.00 | 0.484 | 0.811 | 0.771 | 1.000 | 5 | 4.00 | 0.328 | 0.820 | 0.793 | 0.079 |
| GQ302646 | 9 | 7.96 | 0.643 | 0.888 | 0.859 | 0.000 | 9 | 7.97 | 0.806 | 0.903 | 0.879 | 0.000 | 9 | 8.86 | 0.793 | 0.854 | 0.820 | 1.000 | 11 | 10.00 | 0.161 | 0.914 | 0.891 | 0.000 | 9 | 8.02 | 0.597 | 0.960 | 0.954 | 0.000 |
| GQ302647 | 4 | 3.00 | 0.393 | 0.788 | 0.737 | 0.000 | 2 | 1.86 | 0.226 | 0.508 | 0.375 | 0.000 | 10 | 9.89 | 0.241 | 0.895 | 0.867 | 0.000 | 3 | 2.79 | 0.419 | 0.648 | 0.560 | 1.000 | 4 | 3.75 | 0.319 | 0.851 | 0.831 | 0.093 |
| GQ302656 | 5 | 4.00 | 0.607 | 0.721 | 0.662 | 1.000 | 3 | 2.49 | 0.548 | 0.665 | 0.579 | 0.022 | 3 | 2.89 | 0.586 | 0.636 | 0.572 | 0.000 | 3 | 2.89 | 0.516 | 0.529 | 0.466 | 0.022 | 5 | 4.29 | 0.563 | 0.852 | 0.832 | 0.044 |
| HCM01 | 9 | 7.00 | 0.214 | 0.892 | 0.865 | 0.000 | 9 | 8.89 | 0.194 | 0.917 | 0.894 | 0.000 | 8 | 7.00 | 0.207 | 0.909 | 0.885 | 0.000 | 2 | 1.98 | 0.355 | 0.506 | 0.374 | 0.000 | 9 | 7.75 | 0.244 | 0.942 | 0.934 | 0.000 |
| HCM02 | 5 | 4.00 | 0.643 | 0.788 | 0.737 | 1.000 | 4 | 3.68 | 0.581 | 0.747 | 0.685 | 0.004 | 9 | 8.02 | 0.517 | 0.886 | 0.857 | 0.000 | 4 | 3.00 | 0.387 | 0.690 | 0.628 | 1.000 | 5 | 4.38 | 0.529 | 0.880 | 0.864 | 0.891 |
| HCM08 | 7 | 5.96 | 0.214 | 0.933 | 0.910 | 0.000 | 9 | 8.78 | 0.387 | 0.914 | 0.892 | 0.000 | 7 | 6.57 | 0.345 | 0.889 | 0.862 | 0.000 | 8 | 7.67 | 0.290 | 0.940 | 0.920 | 0.000 | 7 | 6.69 | 0.311 | 0.971 | 0.965 | 0.000 |
| HCM11 | 6 | 5.89 | 0.464 | 0.756 | 0.711 | 0.000 | 7 | 6.57 | 0.290 | 0.852 | 0.818 | 0.000 | 7 | 6.38 | 0.414 | 0.865 | 0.832 | 0.000 | 6 | 6.00 | 0.355 | 0.821 | 0.779 | 1.000 | 6 | 4.89 | 0.378 | 0.916 | 0.906 | 0.000 |
| HCM13 | 5 | 4.89 | 0.536 | 0.746 | 0.690 | 0.015 | 3 | 2.79 | 0.484 | 0.721 | 0.658 | 0.002 | 11 | 10.92 | 0.414 | 0.905 | 0.880 | 0.000 | 7 | 6.89 | 0.484 | 0.876 | 0.846 | 0.001 | 5 | 4.25 | 0.479 | 0.920 | 0.910 | 0.000 |
| HCM14 | 4 | 3.67 | 0.429 | 0.694 | 0.624 | 0.011 | 5 | 4.89 | 0.452 | 0.787 | 0.744 | 0.000 | 10 | 8.96 | 0.276 | 0.917 | 0.893 | 0.000 | 3 | 2.18 | 0.613 | 0.743 | 0.682 | 0.006 | 4 | 3.46 | 0.445 | 0.899 | 0.887 | 0.000 |
| HCM29 | 11 | 10.81 | 0.286 | 0.920 | 0.896 | 0.000 | 7 | 6.99 | 0.355 | 0.764 | 0.716 | 0.000 | 9 | 7.99 | 0.276 | 0.910 | 0.885 | 0.000 | 7 | 6.29 | 0.355 | 0.861 | 0.828 | 0.000 | 11 | 9.85 | 0.319 | 0.947 | 0.941 | 0.000 |
| MP19 | 10 | 8.90 | 0.321 | 0.896 | 0.868 | 0.000 | 6 | 5.89 | 0.452 | 0.774 | 0.726 | 0.002 | 8 | 7.93 | 0.310 | 0.822 | 0.781 | 0.000 | 11 | 10.10 | 0.419 | 0.898 | 0.874 | 1.000 | 10 | 8.72 | 0.378 | 0.912 | 0.901 | 0.021 |
| APS28 | 4 | 3.90 | 0.321 | 0.847 | 0.811 | 0.000 | 8 | 7.99 | 0.323 | 0.851 | 0.817 | 0.000 | 10 | 9.99 | 0.345 | 0.905 | 0.879 | 0.000 | 8 | 7.71 | 0.355 | 0.833 | 0.796 | 0.000 | 4 | 3.70 | 0.336 | 0.956 | 0.950 | 0.000 |
| APS45 | 18 | 16.62 | 0.714 | 0.948 | 0.927 | 0.007 | 16 | 14.90 | 0.581 | 0.941 | 0.921 | 0.000 | 6 | 5.79 | 0.724 | 0.943 | 0.922 | 0.000 | 18 | 19.85 | 0.581 | 0.943 | 0.924 | 0.000 | 18 | 16.75 | 0.647 | 0.981 | 0.976 | 0.000 |
| Mean | 7.600 | 6.758 | 0.455 | 0.835 | 0.795 | | 7.067 | 6.777 | 0.417 | 0.803 | 0.756 | | 8.267 | 7.743 | 0.412 | 0.868 | 0.836 | | 7.400 | 6.861 | 0.415 | 0.791 | 0.744 | | 7.600 | 6.750 | 0.424 | 0.918 | 0.907 | |

PY, Poyang Lake; DT, Dongting Lake; HZ, Hung-tse Lake; AQ, Anqing Lake.

**Table 2** Pairwise $F_{ST}$ (below the diagonal) and the number of migrants per generation, $N$m (above the diagonal) among four *Lamprotula leai* stocks estimated from 15 microsatellite loci

|  | PY | DT | HZ | AQ |
|---|---|---|---|---|
| PY |  | 1.46 | 3.18 | 1.47 |
| DT | 0.146* |  | 1.75 | 1.87 |
| HZ | 0.073* | 0.125* |  | 1.52 |
| AQ | 0.146* | 0.118* | 0.142* |  |

PY, Poyang Lake; DT, Dongting Lake; HZ, Hung-tse Lake; AQ, Anqing Lake. *: $P<0.05$.

**Table 3** AMOVA of 15 microsatellites in the four *Lamprotula leai* stocks

| Source of variation | df | Sum of squares | Variance components | Percentage of variation |
|---|---|---|---|---|
| Among stocks | 3 | 187.47 | 0.90 | 12.56* |
| Among individuals within stocks | 115 | 1065.96 | 3.04 | 42.76* |
| Within individuals | 119 | 378.50 | 3.18 | 44.68* |
| Total | 237 | 1631.93 | 7.12 | 100 |

*: $P<0.05$.

**Table 4** Nei's unbiased genetic identity (above diagonal) and genetic distance (below diagonal)

|  | PY | DT | HZ | AQ |
|---|---|---|---|---|
| PY |  | 0.287 | 0.563 | 0.361 |
| DT | 0.890 |  | 0.398 | 0.574 |
| HZ | 0.438 | 0.578 |  | 0.298 |
| AQ | 0.672 | 0.192 | 0.768 |  |

PY, Poyang Lake; DT, Dongting Lake; HZ, Hung-tse Lake; AQ, Anqing Lake.

### Hardy-Weinberg equilibrium

A total of 12 loci significantly deviated from HWE after Bonferroni correction. Departure from HWE can be attributed to random genetic drift, inbreeding, and null alleles which contribute to heterozygote deficiency (Nei, 1987; Zheng et al, 2009). Using Micro-checker, we found five loci including GQ302635, *HCM08*, *HCM29*, *MP19* and *APS45* with null alleles. This phenomenon is not unusual when analyzing conservation genetics using cross-amplification methods (Harper et al, 2003). However, each locus which departured from HWE can amplify at least one allele in all samples which means that the frequency of null alleles were not enough to affect the analysis (Goodman et al, 2001). In this study, $F_{is}$ was positive and both $H_O$ and $H_E$ had large differences, suggesting an ongoing inbreeding among the tested stocks of *L. leai*. If accurate, then inbreeding is likely a critical factor accounting for departure from HWE, highlighting the need for better management and planned breeding.

### Feasibility of cross-species amplification in shellfish

To date, 18 microsatellite loci for *L. leai* have been screened, but developing further microsatellite loci is essential for further more targeted research and for more cross-species applications. The primers derived from the relatively conservative flanking sequences can be used for amplification across species. Some studies in shellfish have shown the feasibility of cross-species amplification method. For instance, Xu et al (2011) isolated and characterized 18 loci in *L. leai*, 10 of which were successfully amplified in three *H. cumingii* populations. Wang et al (2006) confirmed that 13 loci from 32 polymorphic microsatellite primers of *Crassostrea gigas* can amplify specific products in *H. cumingii*. Hai et al (2009) indicated that 17 primers of *Perca schrenkii* were amplified in *P. fluviatilis* and *P. flavescens*, among which 10 pairs showed versatility in the same genus. The microsatellite loci isolated in this study may then provide useful information for further inquiries into shellfish genetic resource information collecting using cross-amplification.

### CONCLUSION

In conclusion, the four tested stocks of *L. leai* examined in this study showed both high genetic diversity and medium genetic differentiation, but we also encountered evidence suggesting ongoing inbreeding among the stocks. These results may be related to the life habits and reproductive characteristics of *L. leai*, or to the currently employed methods of farming and aquaculture. Given this result, genetic management should be carried out in order to maintain the genetic integrity before the situation deteriorates (Jia et al, 2012). Based on our results, we suggest that specimens from Hung-tse Lake may be viable parents for systematic breeding and hybrid efforts that may result in offspring that could offset the apparent inbreeding and maintain the genetic diversity we observed. On the whole, however, our results and methodology may be useful in identifying growth traits associated markers, constructing of genetic linkage maps, and marker-assisted breeding of *L. leai*.

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