Development and usage of microsatellite markers for population analysis of *Corbicula japonica* in Japan

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**Abstract:** Six novel microsatellite markers were developed in *Corbicula japonica* and used (along with an additional locus that was previously reported for another species) for population analysis of this species in Japan. The samples were collected from 4 local populations: Lake Abashiri and Lake Oikamanai in Hokkaido, Lake Ogawara in Aomori Prefecture, and Lake Shinji in Shimane Prefecture. The genetic diversity of the Shinji population was lower than that of the other 3 populations. Pair-wise $F_{ST}$ analysis revealed significant genetic differentiation among all of the local populations and suggested two groups: northern (Abashiri, Oikamanai, and Ogawara) and southern (Shinji). Bayesian clustering analysis also supported this grouping, and analysis of molecular variance showed a hierarchical population structure for the two groups when genetic differentiation was calculated using $R_{ST}$. It is concluded that these local populations are genetically different from each other and that there is marked differentiation between geographically distant populations. These results demonstrate that these new microsatellite markers are useful for estimating genetic diversity and population structure, and should aid future research on effective aquaculture systems and sustainable fisheries for this species.

**Key words:** *Corbicula japonica*, genetic diversity, microsatellite, population structure

**Introduction**

Population genetics in aquatic species provides essential knowledge for conservation and bioresource management, including information about genetic differentiation that indicates dispersal pathways between localities, and temporal changes in genetic diversity during demographic processes (Ciftci & Okumus 2002, Policansky & Magnuson 1998, Roldan et al. 2000, Ward 2000).

The clam *Corbicula japonica* (Prime 1864), distributed in brackish water lakes and the tidal flats of rivers from Sakhalin Island to the south of Japan, is one of the most important resources of inland water fisheries in Japan (Nakamura 2000) and also supports nitrogen cycles in natural ecosystems in brackish lakes and rivers (Nakamura et al. 1998). In 2010 in Japan, the catch of *Corbicula* spp. amounted to 11,189 t; *C. japonica* constituted the vast majority of this catch (Statistics Department of Ministry of Agriculture 2011). Recently, the catch of *C. japonica* has decreased because of natural and anthropogenic disturbances such as overfishing and operation of estuary barrages (e.g. in Nagara River, Gifu; Yamauchi 2002). Lake Shinji in Shimane Prefecture has maintained the most exploited population of *C. japonica* in Japan, but the catch of *C. japonica* drastically decreased because of overpopulation, lowered tolerance in anoxic conditions (Aizaki et al. 2001), and salinity changes caused by heavy rain (Fujihara et al. 2011). The level of genetic diversity requires monitoring to evaluate the vulnerability of such populations.

The decline of *Corbicula* resources has caused other problems such as the high costs of importing exotic *Corbicula* spp., haphazard stocking, and mislabeling. *Corbicula fluminea* (Müller 1774) has been imported to Japan as a food resource, and has sometimes been sold as *C. japonica*, which are more highly valued for their flavour than *C. fluminea*. When they were released as stock into rivers and lakes, some of them escaped to other water systems, and there was an increasing risk of hybridization with, or replacement of, native *Corbicula* spp. (Ishibashi & Komaru 2003). Identification of genetic species with DNA markers
It is necessary to survey and resolve these problems. Domestic mislabeling of *C. japonica* in Japan can also be traced using DNA markers. Furthermore, genetic information is necessary for effective resource recovery by restocking because the clam seed that is genetically close to the natural population is expected to have a higher survival rate after transplantation, and to have a lower impact at the genetic level than exchanging seed between genetically distant populations.

Fujihara et al. (2011) developed a mitochondrial DNA marker that could identify the origin of a *C. japonica* sample, effectively distinguishing two population groups: northern (Lake Ogawara) and southern (Lake Shinji, Sashimi River, Lake Jinzai, Hinuma River, Ibi River, and Kiso River). However, molecular tools for detailed population analysis, such as microsatellite markers, have not been available. A microsatellite, which is codominant and biparentally inherited, can be used to genetically discriminate individuals and populations because of its high level of variability (see, for example, Freeland 2005). Recently, several studies have been published using microsatellite markers for population analysis of other clam species, such as *Panopea abrupta* (Bentzen 2004), *Mactra chinensis* (Ni et al. 2010), and *Ensis siliqua* (Varela et al. 2012), suggesting the high utility of developing microsatellites for population analysis in *C. japonica*.

The purpose of the present study was to develop and practically implement the use of microsatellite markers for Japanese populations of *C. japonica*. The results of population analyses are used in discussing the availability of markers, genetic diversity within populations, and differentiation among populations.

### Materials and Methods

**Samples**

Genetic analysis was performed using 164 individuals from 4 localities in Hokkaido and Honshu, Japan (see Table 1 and Fig. 1). The samples were collected from populations in Lake Abashiri (32.3 km², in Hokkaido), Lake Oikamanai (1.7 km², in Hokkaido), Lake Ogawara (63.2 km², in Aomori Prefecture), and Lake Shinji (79.1 km², in Shimane Pref). Among these lakes, the largest catch of *Corbicula* spp. in a commercial fishery in 2006 was in Lake Shinji (ca. 6393 t), followed by Lake Ogawara (1545 t) and Lake Abashiri (788 t; Statistics Department of the Ministry of Agriculture 2009). In Lake Oikamanai, the commercial fishery of *Corbicula* has been conducted on just 1 day per year, with very few clams caught. On that day, they take only extremely large clams, with some shell lengths exceeding 5 cm (Sonoda et al. 2009, Tanaka et al. 2010).

**DNA extraction**

A small piece of tissue was isolated from the foot muscle of each individual and fixed in 99% ethanol at room temperature until used for DNA extraction. Genomic DNA was extracted from 20–50 mg of muscle tissue with a Pure Gene Kit (Qiagen, Tokyo, Japan) or DNeasy Blood & Tissue Kit (Qiagen) in accordance with the manufacturer’s protocol. Extracted DNA was dissolved in 200 µL of elution buffer (Buffer AE; Qiagen Kit).

**Development and characterization of microsatellite markers**

Essentially, the procedures followed those described by Hamilton et al. (1999) and by Glenn and Schable (2005),
using streptavidin-coated magnetic particles and biotinylated oligonucleotide probes. After enrichment for repeat regions and nucleotide sequencing, 20 pairs of PCR primers were designed and PCR amplification of each primer pair was tested using the genomic DNA of 8 individuals from Lake Abashiri. The PCR amplification procedure followed the manufacturer's instructions, using a Qiagen Multiplex PCR Kit (Qiagen) in 15 μL reactions that included 0.5 μM of each primer and ca. 50 ng of genomic DNA. The thermal profile included pre-cycling denaturation at 95°C for 15 min; 35 cycles of denaturation at 95°C for 30 s, annealing at a temperature suitable for each locus (see Table 2) for 60 s, and extension at 65°C for 30 s; and a post-cycling extension step at 72°C for 10 min. The forward primers of the primer pairs that performed well in amplification were then fluorescently labeled with 6-FAM, NED, PET, or VIC. Using these fluorescent primer sets, PCR was carried out in the same manner as above except that the post-cycling extension step was at 72°C for 30 min to promote the addition of adenine at the 3′-end of all PCR products. The ensuing PCR products were sized using a CEQ8000 DNA sequencer (Beckman Coulter, Fullerton, CA). Finally, 6 polymorphic loci were selected for further analyses and their sequence data were deposited in GenBank (accession nos. AB671263–AB671268).

In the following analysis, these 6 newly developed microsatellite markers were used as well as an additional, previously reported marker, CIC01 (Pigneur et al. 2011), which could be amplified by PCR and exhibited length polymorphism in our samples. The number of alleles, expected and observed heterozygosities, departure from the equilibrium of these 7 markers were assessed with Genepop (Raymond & Rousset 1995, Rousset 2008). Allelic richness, which is the corrected allele numbers for the minimum sample size (viz. 36 individuals) was quantified with FSTAT ver. 2.9.4 (Goudet 1995). Potential genotyping errors, such as allelic dropouts, stuttering alleles, or null alleles, were monitored using the Micro-Checker ver. 2.2.3 (Van Oosterhout et al. 2004).

**Population genetic analysis**

Population structure and genetic connectivity between local populations were assessed in three ways: pair-wise FST and RST values, calculated by Arlequin ver. 2.00 (Schneider et al. 2000); Bayesian clustering analysis implemented in Structure ver. 2.1 (Pritchard et al. 2000, Falush et al. 2003; ref. also protocol ver. 2.3.3); and analysis of molecular variance (AMOVA) by Arlequin (Schneider et al. 2000).

The FST and RST values were calculated as indices of genetic distance between populations, and significant deviation from zero of FST values was tested at α=0.00167 (0.01/6) after Bonferroni correction. Slatkin’s linearized FST methods (Slatkin 1995) was then used to convert all FST and RST values to positive values. The genetic distance between populations was two-dimensionally visualized by multidimensional scaling (MDS) plots with the statistical software R ver. 2.9.0 (R Development Core Team), based on linearized FST and RST values between populations.

In the STRUCTURE analysis, likelihoods for each number of assumed source populations (K) were calculated from allele frequency data, and each individual was assigned to a plausible population. In the simulation, either an admixture or non-admixture ancestry model was chosen, along with a correlated or independent allele frequency model. All four model sets were tested: (A) admixture and correlated, (B) admixture and independent, (C)
non-admixture and correlated, and (D) non-admixture and independent. Each run was performed with a burn-in period of 100,000 simulations followed by 100,000 Markov Chain Monte Carlo simulations. For each $K$ (2, 3, 4), 10 iterations were performed. The most suitable model and the most plausible number for $K$ were selected by the largest likelihood value ($\ln P(X|K)$) and the smallest variance of $\ln P(X|K)$. The $Q$ value for each individual was calculated as an index of the probability that the individual was derived from the assumed population. The average $Q$ for each local population was calculated as an index of the probability that the population belonged to, or was derived from, the assumed population.

Based on the results of $F_{ST}$, $R_{ST}$, and STRUCTURE analyses, the 4 populations were clustered into two groups for AMOVA: (1) Abashiri, Oikamanai, and Ogawara, and (2) Shinji. Molecular variance was estimated in two ways in AMOVA (by $F_{ST}$ and $R_{ST}$) with 1,000 permutations per run.

**Results**

**Characterization of microsatellite markers**

The characteristics of the microsatellite markers used in this study are summarized in Table 2. The number of alleles in the samples analyzed ranged from 3 in C37 to 68 in C84, and expected heterozygosities ranged from 0.14 in C37 to 0.98 in C277. When all individuals were pooled as a single population, the deviation from HWE was significant in 5 of 7 loci, indicating the existence of a population subdivision. Diversity indices in each population are shown in Table 3. The mean values for allelic richness and expected and observed heterozygosities were the lowest in the Shinji population. A significant deviation from HWE was observed for C277, C18, and CIC01 in the Abashiri population; C18 in that from Oikamanai; C277 and CIC01 from Ogawara; and C102, C84, and C277 from Shinji. The possibility of null alleles at these loci was suggested by analysis with Micro-Checker, although the program revealed no evidence for scoring error due to stuttering or for large-allele dropout at any of the loci in any of the populations.

**Population genetic analysis**

Pair-wise $F_{ST}$ and $R_{ST}$ analyses indicated genetic differentiation between local populations. $F_{ST}$ values were significant in all pairs of populations, but $R_{ST}$ values were not significant in the pair consisting of the Ogawara and Oikamanai populations. MDS plots based on $F_{ST}$ and $R_{ST}$ values are shown in Fig. 2. Both methods indicated that the Abashiri, Oikamanai, and Ogawara populations are closely related to each other, whereas the Shinji population was found to be distant from the other three.

In the STRUCTURE analysis, model C (non-admixture ancestry model and correlated allele-frequency model) was chosen because it had higher mean likelihood-values ($\ln P(X|K)$) and lower variance of $\ln P(X|K)$ than the other models. Using model C, the mean values of $\ln P(X|K)$ in 10 iterations were $-5783.0$, $-5666.5$, and $-5587.0$ for $K=2$, 3, and 4, respectively, suggesting that the most plausible

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**Table 3.** The number of alleles ($N_e$), allelic richness ($A_e$) and expected and observed heterozygosity ($H_e$ and $H_o$) at each locus in each sample.

|       | C102 | C84 | C277 | C19 | C18 | C37 | CIC01 | Mean |
|-------|------|-----|------|-----|-----|-----|-------|------|
| Abashiri |  | 15  | 34  | 23  | 27  | 24  | 3     | 18.86|
|        | $n=45$ | 13.67 | 31.29 | 21.52 | 25.05 | 22.45 | 2.79  | 5.80 | 17.51|
|        | $H_e$ | 0.77 | 0.97 | 0.93 | 0.96 | 0.94 | 0.09  | 0.74 | 0.77 |
|        | $H_o$ | 0.62 | 0.91 | 0.64* | 1.00 | 0.76* | 0.09  | 0.49* | 0.64 |

| Oikamanai |  | 17  | 40  | 29  | 26  | 19  | 6     | 19.86|
|          | $n=41$ | 15.99 | 37.61 | 28.26 | 25.29 | 18.13 | 2.00  | 5.99 | 19.04|
|          | $H_e$ | 0.72 | 0.97 | 0.97 | 0.95 | 0.93 | 0.22  | 0.79 | 0.79 |
|          | $H_o$ | 0.58 | 0.85 | 0.83 | 1.00 | 0.78* | 0.24  | 0.66 | 0.71 |

| Ogawara  |  | 26  | 42  | 18  | 24  | 25  | 2     | 20.71|
|          | $n=42$ | 24.04 | 38.60 | 17.35 | 23.56 | 24.04 | 2.00  | 7.86 | 19.63|
|          | $H_e$ | 0.91 | 0.98 | 0.93 | 0.96 | 0.96 | 0.17  | 0.77 | 0.81 |
|          | $H_o$ | 0.78 | 0.95 | 0.55* | 0.93 | 0.90 | 0.19  | 0.55* | 0.69 |

| Shinji   |  | 25  | 25  | 11  | 20  | 25  | 2     | 15.86|
|          | $n=36$ | 25.00 | 25.00 | 11.00 | 20.00 | 25.00 | 2.00  | 5.00 | 16.14|
|          | $H_e$ | 0.96 | 0.94 | 0.60 | 0.93 | 0.95 | 0.06  | 0.74 | 0.74 |
|          | $H_o$ | 0.72* | 0.44* | 0.36* | 1.00 | 0.92 | 0.06  | 0.56 | 0.58 |

| Total    |  | 37  | 68  | 36  | 38  | 34  | 3     | 32.00|
|          | $n=164$ | 0.89 | 0.97 | 0.98 | 0.96 | 0.94 | 0.14  | 0.77 | 0.81 |
|          | $H_e$ | 0.67* | 0.80* | 0.60* | 0.98 | 0.84* | 0.15  | 0.56* | 0.66 |

*Indicates significant deviation from HWE at $p<0.01$ levels after Bonferroni correction.
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number for \( K \) is 4. However, variances of \( \ln P(X|K) \) were 315.1, 716.9, and 755.9 for \( K=2, 3, \) and 4, respectively, suggesting that the most plausible value for \( K \) is 2. \( Q \) was then calculated for \( K=2, 3, \) and 4 (Fig. 3). All schematics indicated that the Shinji population is markedly different from the others. When \( K \) was assumed to be 3 or 4, each individual in the Abashiri, Oikamanai, and Ogawara populations appeared to have a similar degree of admixture, indicating that the separation among them is not real, as indicated in the protocol for STRUCTURE. Consequently, the results indicated the existence of 2 groups, confirming the \( F_{ST} \) and \( R_{ST} \) analyses. The \( Q \) value for each population is shown in Table 4, indicating that the Abashiri, Oikamanai, and Ogawara populations are derived from a single ancestor population but that the origin of the Shinji population was different.

**Table 4.** \( Q \) values indicating the proportion of assigned membership to each assumed population (pop 1 and pop 2).

| Population  | pop 1 | pop 2 |
|-------------|-------|-------|
| Abashiri    | 0.943 | 0.057 |
| Oikamanai   | 0.733 | 0.267 |
| Ogawara     | 0.891 | 0.109 |
| Shinji      | 0.031 | 0.969 |

Values in boldface indicate higher probabilities.

**Table 5.** The results of analysis of molecular variance (AMOVA) assuming two groups: north (Abashiri, Oikamanai, and Ogawara) and south (Shinji).

| Source of variation | % of variation \( \Phi_{ST} \) | \( p \) |
|---------------------|-----------------------------|-------|
| Distance=\( F_{ST} \) Among groups | 4.37 | 0.045 | 0.248 |
| Among populations within groups | 2.16 | 0.022 | 0.000 |
| Within populations | 93.47 | 0.000 |
| Distance=\( R_{ST} \) Among groups | 10.75 | 0.107 | 0.000 |
| Among populations within groups | 6.14 | 0.069 | 0.000 |
| Within populations | 83.11 | 0.000 |

Fig. 2. Multidimensional scaling (MDS) plots based on \( F_{ST} \) and \( R_{ST} \) values between populations. The Shinji population appears to be distant from the other three populations.

Fig. 3. The proportions of assigned membership to each assumed \( K \) population for each individual. Each vertical bar represents 1 individual. The vertical lengths of bars in different colors within each individual indicate the probability of belonging to the assumed populations.
The hierarchical population structure of the two groups is also supported by AMOVA (Table 5) when an \( F_{ST} \)-based calculation was employed. However, the structure was not significant in \( R_{ST} \)-based calculations because of the high variance within each population.

**Discussion**

The present study documents microsatellite markers developed for population genetic studies of *C. japonica* and demonstrates their practical application. The number of alleles encountered in the samples studied here ranged from 3 to 68, and the expected heterozygosity ranged from 0.14 to 0.98, indicating that these microsatellite markers were polymorphic and available for population analysis. Markers *C37* and *CIC01*, which demonstrated less heterozygosity than the others, are probably useful for genetic analyses at the species level. Marker *CIC01* was developed to discriminate the lineage in invasive *Corbicula* clams, including multiple species in Western Europe (Pigneur et al. 2011). In their analysis, allele size ranged from 173 to 179 bp in invasive clams, probably including *C fluminea* and *C. fluminalis*; whereas, in the present study, allele size ranged from 156 to 168 bp in *C. japonica*, indicating the potential of *CIC01* for species identification. However, the hypervariable loci (*C102*, *C84*, *C277*, *C19*, and *C18*) may be useful for kinship and paternity analysis to elucidate the breeding patterns of this species.

Genetic diversity indices (i.e. allelic-richness and expected and observed heterozygosity) were low in the Shinji population, as shown in Table 3. Although the potential existence of null alleles in *C102*, *C84*, and *C277* in Shinji was suggested by Micro-Checker analysis, the occurrence of null alleles is considered to be a rare phenomenon because amplification of *C102*, *C84*, and *C277* did not fail in any individual in the Shinji population in our analysis. Thus, the deviation from HWE may represent skewed genetic structure in this lake. Taking into account the large catch in Lake Shinji, this lake may have the largest population of *C. japonica* in Japan. Genetic variation is correlated with population size (Frankham 1996), so genetic diversity in the Lake Shinji population was expected to be higher than that in the other populations. However, the commercial fishery of this lake has a long history, and heavy catch pressure may have caused a severe decline in population size on several past occasions, decreasing genetic diversity as a bottleneck effect. Moreover, environmental changes, including the results of artificial effects and extraordinary climatic events may lower population size and genetic diversity in this lake, as demonstrated by the recent population decline described by Aizaki et al. (2001) and Fujihara et al. (2011).

Furthermore, even if the null alleles in the Shinji population are taken into account, the genetic data were still informative in population analysis, indicating the distinction between the Shinji population and the other three populations. More specifically, a significant deviation from HWE was not detected for *C102* and *C84* in the northern populations (Abashiri, Oikamani, and Ogawara), suggesting that null alleles were not present in these populations. The null allele represents a lack of the locus in the genome or nucleotide substitution at the primer region, indicating genetic differentiation from other ‘normal’ alleles in certain individuals. Thus, the finding that null alleles occurred only in the Shinji population indicate genetic differentiation of this population from the others.

The results of this study revealed a northern and a southern group, which is essentially congruent with the analysis of *C. japonica* mitochondrial DNA described by Fujihara et al. (2011). The samples investigated in their study included clams from Lake Shinji and Lake Ogawara, the same locations from where samples for the present study were collected. The Shinji and Ogawara populations belonged to the southern and northern groups, respectively, in both studies. In addition, the microsatellite analysis in the present study revealed genetic differentiation among each of the component populations within the northern group. The \( F_{ST} \) values were not particularly high because of the high variability within each population, but significant differentiation was demonstrated by the \( p \)-values obtained. Further investigation with more samples and combined application of mitochondrial and other nuclear DNA markers is expected to clarify the population structure of *C. japonica* in Japan in more detail.

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