

# Genetic Variability and Reproduction Structure of *Corbicula japonica* in Major Fishing Brackish Lakes in Japan

Tsudzumi Mito<sup>1</sup>, Tomomi Tanaka<sup>2</sup>, Futoshi Aranishi<sup>1,2,3</sup>

<sup>1</sup>United Graduate School of Agricultural Sciences, Tottori University, Tottori, Japan

<sup>2</sup>Center for the Promotion of Project Research, Shimane University, Matsue, Japan

<sup>3</sup>Coastal Lagoon Research Center, Shimane University, Matsue, Japan

Email: [aranishi@soc.shimane-u.ac.jp](mailto:aranishi@soc.shimane-u.ac.jp)

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## Abstract

*Corbicula japonica* is the best-known bivalve inhabiting widely in brackish estuaries and lakes in Japan. Although this species has been most commercially important species of inland fisheries in Japan, the gradual decline in its production over 40 years caused not only economic problems in fishery industry but also ecological disturbances in biodiversity conservation. The aim of this study was to evaluate the reproduction structure of *C. japonica* populations in major fishing brackish lakes based on the genetic diversity inferred by mitochondrial DNA sequence analysis of the cytochrome oxidase *c* subunit I gene. Of a total of 188 *C. japonica* individuals collected in Lakes Shinji, Jusan, Ogawara and Abashiri, 25 haplotypes were obtained, and only the haplotype HT01 was apparent with relatively high abundance in all lakes. Minimum spanning network analysis of haplotypes showed different population structures between Lake Shinji and Lakes Jusan, Ogawara and Abashiri. In addition, pairwise population genetic distance  $F_{ST}$  and  $\Phi_{ST}$  values were significantly higher in Lake Shinji than Lakes Jusan, Ogawara and Abashiri. The mismatch distribution analysis showed unimodal profile for Lakes Jusan and Ogawara and bimodal profile for Lakes Shinji and Abashiri. Those results indicate a recent population expansion in all lakes, and Lakes Shinji and Abashiri and Lakes Jusan and Ogawara maintained continuous reproduction structure and suffered to rapid population growth, respectively.

## Keywords

*Corbicula japonica*, Brackish Lakes, Genetic Diversity, Reproduction Structure, Cytochrome Oxidase *c* Subunit I Gene

## 1. Introduction

Species belonging to the genus *Corbicula* attain an almost worldwide distribution and inhabit in estuaries, lakes, and rivers [1]-[3]. In Japan, 3 endemic *Corbicula* species are reproductively and developmentally heterogeneous [4]-[7]. Although *C. leana* and *C. sandai* appear fresh waters only in Japan, *C. japonica* inhabits blackish waters throughout eastern Asia from Kyushu Island in Japan to Sakhalin Island in Russia between approximately 27°N and 55°N [8].

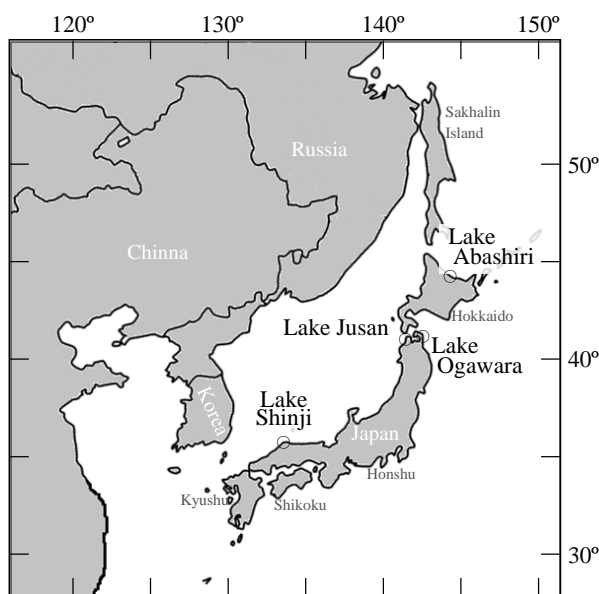
*C. japonica* has been one of the most important species for inland fisheries in Japan [9], and occupied 99% of the total catch of *Corbicula* [8]. The catch of *Corbicula* from 2002 to 2011 reached average 12,000 tons, which corresponded to approximately 30% of the total catch of inland fisheries [10]. As fisheries of *C. japonica* have so far prospered in a number of lakes and marshes, Lake Shinji in Shimane Prefecture, Lakes Jusan and Ogawara in Aomori Prefecture, Lake Abashiri in Hokkaido Prefecture, and Kiso Three Rivers in Mie, Gifu and Aichi Prefectures are recent major fishing areas [11]. However, the catch of *C. japonica* decreased since 1970s due to overfishing, habitat destruction and coastal pollution caused by reclamation work and estuary barrage [12]. For the purpose of an effort to promote stock restoration and increase fisheries production, seedlings and/or adults of *C. japonica* suffered to frequent transplantation among fishing areas. In addition, a bulk of anonymous *Corbicula* individuals was successively imported from eastern Asia [12]. It has been therefore concerned with the genetic disturbance of *C. japonica* in Japan, but its genetic diversity and reproduction structure are still not well understood.

Mitochondrial DNA (mtDNA) has been extensively used for both population genetic and systematic genetic studies on various taxonomic levels [13]. The mtDNA cytochrome *c* oxidase subunit I (COI) gene has often been adopted as a tool for determining the genetic diversity of fisheries valuable bivalves in Japan [14]-[16]. The aim of this study was to evaluate the genetic variability and reproduction structure of *C. japonica* populations among 4 major fishing brackish lakes in Japan using the mtDNA COI gene. The genetic information contributes to stock management of *C. japonica* resources for long-term sustainable fisheries and aquaculture.

## 2. Materials and Methods

### 2.1. Sample Collection

A total of 188 individuals were collected from Lakes Shinji (N = 45), Jusan (N = 48), Ogawara (N = 48), and Abashiri (N = 47) in September 2009 and August 2010 (Table 1, Figure 1). All specimens were boiled, and



**Figure 1.** Map of East Asia at which open circles represent the collection sites of *Corbicula japonica* in Lakes Shinji, Jusan, Ogawara and Abashiri in Japan.

**Table 1.** Shell length and height of *Corbicula japonica* collected in Lakes Shinji, Jusan, Ogawara and Abashiri.

Lake	<i>n</i>	SL mean (mm) ± S.D.	SH mean (mm) ± S.D.
Shinji	45	18.16 ± 2.17	15.87 ± 1.76
Jusan	48	26.73 ± 2.51	23.90 ± 2.24
Ogawara	48	22.34 ± 1.43	20.42 ± 1.31
Abashiri	47	24.28 ± 1.41	22.46 ± 1.27
Total	188	22.94 ± 3.66	20.73 ± 3.44

SL, SH and S.D. represent shell length, shell height and standard deviation, respectively.

adductor muscle or foot tissue were excised from soft tissue and immediately stored at  $-20^{\circ}\text{C}$  until DNA extraction.

## 2.2. DNA Extraction

High-quality total genomic DNA was prepared from small scraps of frozen adductor muscle or foot tissue according to the modified urea-SDS-proteinase K method [17]-[19]. Samples were incubated in the extraction buffer (10 mM Tris-HCl, pH 7.5, 20 mM EDTA, pH 8.0, 1% SDS, and 4 M urea) containing 25  $\mu\text{g}$  proteinase K at  $55^{\circ}\text{C}$ , and 5 M NaCl was then added and mixed. DNA was isolated with phenol-chloroform-isoamyl alcohol and subsequently with chloroform-isoamyl alcohol followed by precipitation with ethanol. DNA pellets were washed with ethanol, dried, and resuspended in 10T0.1E (10 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, pH 8.0).

## 2.3. PCR Amplification

PCR amplification of the mtDNA COI gene was performed in GoTaq Green PCR Master Mix (Promega) containing 2 mM  $\text{MgCl}_2$ , 0.5  $\mu\text{M}$  of each primer, and template DNA in a Techgene thermal cycler (Techne). PCR primers were LCO1490 5'-GGTCA ACAA TCATA AAGAT ATTGG-3' and HCO2198 5'-TAAAC TTCAG GGTGA CAAA AAATC A-3' [20]. PCR protocol consisted of an initial denaturation at  $94^{\circ}\text{C}$  for 2 min, followed by 35 cycles of 1 min at  $94^{\circ}\text{C}$ , 30 sec at  $50^{\circ}\text{C}$ , and 1 min at  $72^{\circ}\text{C}$ , and a final extension at  $72^{\circ}\text{C}$  for 5 min. PCR products were analyzed using a DNA-1000 Reagent Kit (Shimadzu) containing a SYBR Gold Nucleic Acid Gel Stain (Invitrogen) in a MCE-202 MultiNA microchip electrophoresis system (Shimadzu).

## 2.4. Sequence Analysis

Nucleotide sequencing of double strands of PCR product was accomplished using a BigDye Terminator ver. 3.1 Cycle Sequencing Kit (Applied Biosystems) in an automated 3730xl DNA Analyzer (Applied Biosystems). The determined sequences were edited and aligned with CLUSTAL W [21] using MEGA ver. 5.5 [22], followed by submission to GenBank under AB971384-AB971408. Haplotype network based on statistical parsimony was resolved using TCS ver. 1.21 [23]. Haplotype diversity  $h$  and nucleotide diversity  $\pi$  were estimated using Arlequin ver. 3.5 [24]. Pairwise  $F_{ST}$  and  $\Phi_{ST}$  values among sampling localities were calculated using Arlequin ver. 3.5 [24], following which their significance was evaluated by performing a randomization test with 10,000 replications and Bonferroni corrections [25]. Neighbor-joining (NJ) tree with bootstrap analysis was constructed on the basis of the Kimura's 2-parameter model [26] with 10,000 replications using NEIGHBOR in PHYLIP ver. 3.68 [27]. A distance matrix for all pairwise haplotype comparisons was constructed, and the maximum number of mutational differences justified by the parsimony limit of 0.95 was estimated. Mismatch distribution analysis of nucleotide sequences was performed for goodness-of-fit to simulated values of sudden population expansion by parametric bootstrapping with 10,000 replicates using Arlequin ver. 3.5 [24]. Tajima's  $D$  value [28] and Fu's  $F_s$  value [29] for neutrality test were estimated from sequence variations using Arlequin ver. 3.5 [24].

## 3. Results

The average shell length and shell height of *C. japonica* individuals collected in Lakes Jusan, Ogawara and Abashiri ranged from  $22.34 \pm 1.43$  mm to  $26.73 \pm 2.51$  mm and from  $20.42 \pm 1.31$  mm to  $23.90 \pm 2.24$  mm, re-

spectively. In contrast, the respective values of those collected in Lake Shinji were determined to be  $18.16 \pm 2.17$  mm and  $15.87 \pm 1.76$  mm, and relatively small shell size compared with the other lakes (Table 1). Fisheries cooperatives have defined minimum shell size of the catch for stock management in individual lake, and 10, 12, 15 and 14 mm are in Lakes Shinji, Jusan, Ogawara and Abashiri, respectively.

After editing nucleotide sequences of PCR product encoding the mtDNA COI gene, a portion of 556 bp were aligned from 188 individuals collected in 4 lakes, and 24 variable sites and no insertion or deletion were obtained (Table 2). Of 25 detected haplotypes identified to be *C. japonica* (Figure 2), only the haplotype HT01 was shared among all lakes with a high frequency accounting for 78.8% of 188 individuals, and remaining 24 haplotypes from H02 to H25 were unique to individual lake. The haplotype diversity  $h$  values ranged from  $0.2026 \pm 0.0778$  in Lake Abashiri to  $0.6040 \pm 0.0566$  in Lake Shinji (Table 3). Although the frequency of the haplotype H01 was good correlation with the haplotype diversity, the numbers of haplotypes were poor correlation with the  $h$  values. The nucleotide diversity  $\pi$  values of Lakes Jusan, Ogawara and Abashiri ranged from  $0.0591\% \pm 0.0671\%$  to  $0.0601\% \pm 0.0678\%$ , showing relatively low level diversity compared with that value of Lake Shinji to be  $0.2207\% \pm 0.1586\%$  (Table 3).

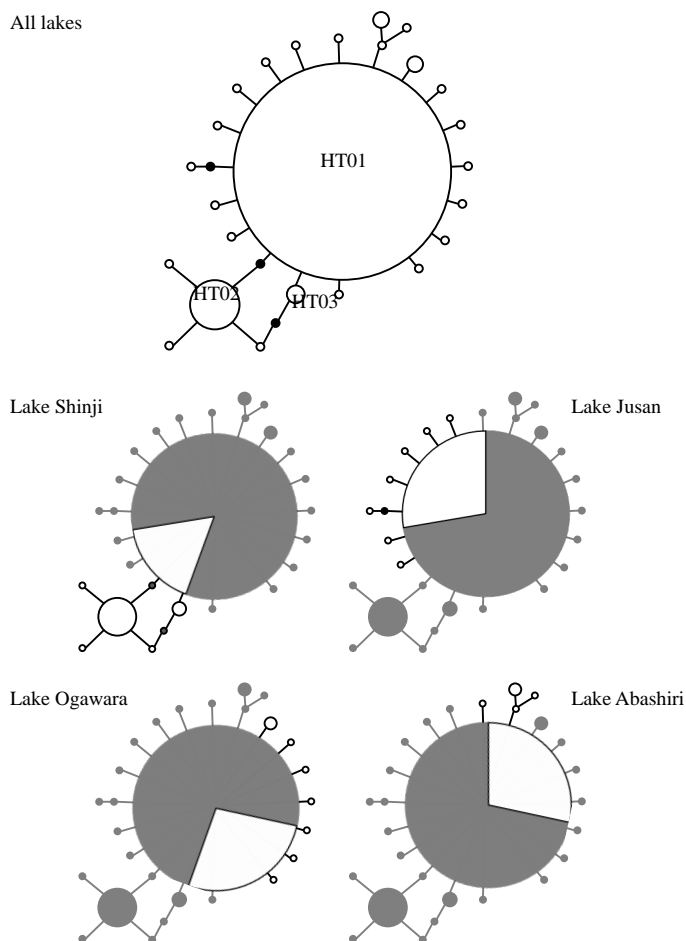
Haplotype network obtained out of 25 haplotypes revealed star-like shape with a partly bush-like clade (Figure 3). Haplotype networks consisted of almost unique haplotypes with 1-2 substitution difference radiating from the haplotype H01, and 3 haplotypes further radiated from the haplotype H02 with 1 substitution difference only in Lake Shinji. They supposedly had a recent history of continuous reproduction, and Lake Shinji was more stable in the reproduction structure than the other lakes. These results were consistent with a markedly high level of the  $\pi$  value in Lake Shinji compared with the other lakes (Table 3).

The pairwise  $F_{ST}$  and  $\Phi_{ST}$  values among 4 lakes ranged from  $-0.0074$  to  $0.2011$  and from  $0.0027$  to  $0.2715$ , respectively, and both values between Lake Shinji and the other lakes were evaluated to be significant (Table 4). In addition, the NJ trees based on the pairwise  $F_{ST}$  and  $\Phi_{ST}$  values separated Lake Shinji from the other lakes (Figure 4).

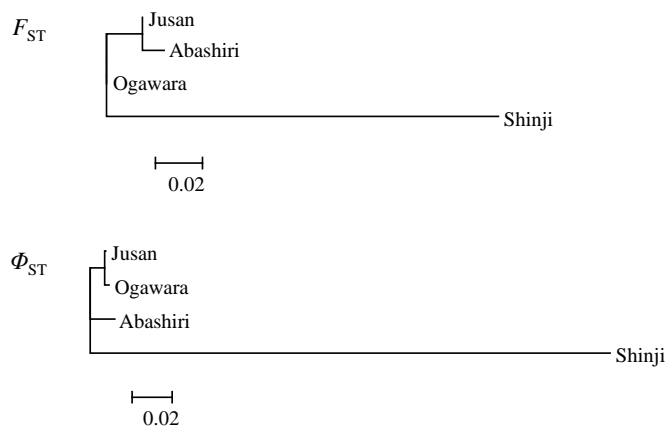
In Lakes Jusan and Ogawara, the mismatch distribution appeared to be unimodal clearly, which closely matched the simulated values, whereas that in Lakes Shinji and Abashiri appeared to be bimodal (Figure 5). The



**Figure 2.** Neighbor-joining tree for H01-H25 haplotypes of *Corbicula japonica* collected in Lakes Shinji, Jusan, Ogawara and Abashiri. Sequences of *C. japonica* (AB988808, AF196271), *C. leana* (AB498810, AF196268), *C. sandai* (AB498811, AF196272), *C. fluminea* (AF196269, AF196270) and *C. fluminalis* (AF457996, AF457997) were used as reference sequences. Bootstrap values higher than 50 are shown at nodes.



**Figure 3.** Minimum spanning haplotype network of *Corbicula japonica* collected in Lakes Shinji, Jusan, Ogawara and Abashiri. White and gray areas indicate haplotype found in individual lake and other 3 lakes, respectively. Solid line and black dot represent single base pair difference among haplotypes and putative mutational step between haplotypes, respectively. Size of circle is proportional to the number of individuals per haplotype.



**Figure 4.** Neighbor-joining trees for *Corbicula japonica* collected in Lakes Shinji, Jusan, Ogawara and Abashiri based on pairwise  $F_{ST}$  values and  $\Phi_{ST}$  values.

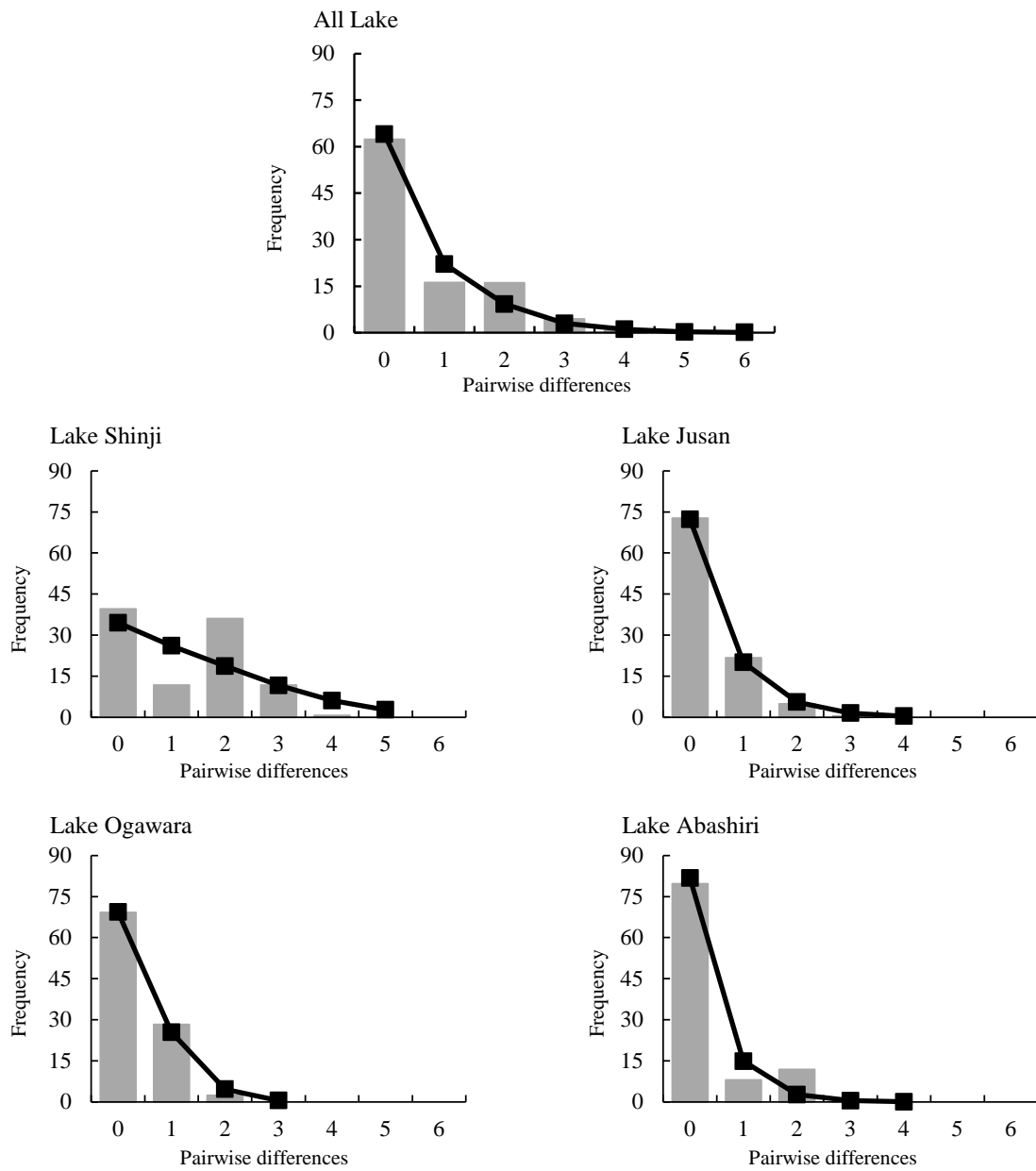
**Table 2.** Nucleotide variations among 25 haplotypes of *Corbicula japonica* collected in Lakes Shinji, Jusan, Ogawara and Abashiri.

	Site																			Lake				Total			
	1	59	61	94	106	145	148	167	183	209	271	283	289	348	355	367	424	466	509	523	526	544	Shinji		Jusan	Ogawara	Abashiri
HT01	T	C	A	C	T	G	C	G	G	C	T	A	G	T	G	T	G	A	A	A	A	G	25	41	40	42	148
HT02	C	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	14				14
HT03	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	2				2
HT04	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	1				1
HT05	C	.	.	.	.	T	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	1				1
HT06	C	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	1				1
HT07	C	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	A	1				1
HT08	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	1				1
HT09	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	1				1
HT10	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	1				1
HT11	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1				1
HT12	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1				1
HT13	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	1				1
HT14	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1				1
HT15	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.		2			2
HT16	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.		1			1
HT17	.	.	.	.	.	.	.	.	.	C	G	.	.	.	.	.	.	.	.	.	.	.		1			1
HT18	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.		1			1
HT19	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.		1			1
HT20	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.		1			1
HT21	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.		1			1
HT22	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	G	.	.				2	2
HT23	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.				1	1
HT24	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.				1	1
HT25	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.				1	1
Total																							45	48	48	47	188

Dot indicates nucleotide identical to that of HT01 sequence.

**Table 3.** Haplotype diversity, nucleotide diversity and number of nucleotide substitution of *Corbicula japonica* collected in Lakes Shinji, Jusan, Ogawara and Abashiri.

Lake	<i>n</i>	Haplotype diversity	Nucleotide diversity (%)	Nucleotide substitution
Shinji	45	0.6040 ± 0.0566	0.2207 ± 0.1586	6
Jusan	48	0.2730 ± 0.0850	0.0601 ± 0.0678	8
Ogawara	48	0.3076 ± 0.0870	0.0598 ± 0.0675	7
Abashiri	47	0.2026 ± 0.0778	0.0591 ± 0.0671	4
Total	188	0.3758 ± 0.0453	0.1171 ± 0.1009	24



**Figure 5.** Mismatch distribution of *Corbicula japonica* collected in Lakes Shinji, Jusan, Ogawara and Abashiri. Vertical bar and solid line represent observed distribution of mismatches and expected distribution of mismatches under the spatial expansion model, respectively.

**Table 4.** Pairwise  $F_{ST}$  values (above the diagonal) and  $\Phi_{ST}$  values (below the diagonal) of *Corbicula japonica* collected in Lakes Shinji, Jusan, Ogawara and Abashiri.

Lake	Shinji	Jusan	Ogawara	Abashiri
Shinji		**0.1675	**0.1529	**0.2011
Jusan	**0.2677		-0.0074	-0.0048
Ogawara	**0.2682	0.0027		0.0007
Abashiri	**0.2715	*0.0194	0.0220	

\*  $p < 0.05$ , \*\*  $p < 0.01$  following Bonferroni correction ( $k = 6$ ).

sum of square deviations (SSD) ranged from 0.0005 in Lake Jusan to 0.0563 in Lake Shinji with no statistical significance ( $p > 0.05$ ), and the Harpending's raggedness index ( $H_{ri}$ ) ranged from 0.2071 in Lake Shinji to 0.5291 in Lake Abashiri with no statistical significance ( $p > 0.05$ ) (Table 5). The Tajima's  $D$  and Fu's  $F_s$  values for all lakes were estimated to be negative, and both values for Lake Shinji were not significant excepting the other lakes (Table 5). These results indicate that *C. japonica* occurred a recent expansion and formed the current geographical distribution in Japan.

#### 4. Discussions

*C. japonica* is an endemic species of East Asia, but little is known about its biological and ecological properties in the native range, because it had been one of the most common species with abundant resources in various parts of Japan. However, its stock level declined over 40 years since 1970s, as its habitat lost due to water pollution and estuary modification [12]. To solve resources reduced, *C. japonica* individuals had been frequently transplanted mainly from Lake Shinji to every fishing area in Japan. These situations strengthened concerns over the genetic loss in *C. japonica* populations. In this study, we selected 4 major fishing brackish lakes with no introduction record to determine the genetic diversity of *C. japonica* in its native range (Figure 1).

Recent developments of genetic identification keys have helped to resolve questions surrounding bivalve genetic studies, and mtDNA markers such as 16S ribosomal RNA (rRNA) gene and COI gene were specifically developed for *Corbicula* species. Komaru et al. [30] reported genetic discrimination of recognized *Corbicula* species based on the 16S rRNA gene, which was verified as a sensitive molecular marker for species identification of marine bivalves [31] [32]. Suzuki et al. [33] reported population genetic structure of *C. japonica* in Japan based on the COI gene, which was also verified as a sensitive molecular marker for population differentiation of marine bivalves [16] [34].

In this study, the mtDNA COI gene was adopted to evaluate the genetic diversity and reproduction structure of *C. japonica* populations in major fishing brackish lakes (Table 2). The genetic diversity in Lake Shinji was higher than that in the other lakes (Table 3), and it therefore provided evidence of highly significant genetic differentiation between Lake Shinji and the other lakes (Figure 4, Table 4). This genetic differentiation was caused by unique population genetic structures on haplotype networks, which were radiated from the haplotypes H01 and H02 in Lake Shinji and from the haplotypes H01 alone in the other lakes (Figure 3). Otherwise, the mismatch distribution analysis indicated unimodal profile for Lakes Jusan and Ogawara and bimodal profile for Lakes Shinji and Abashiri, and the respective profiles show recent histories of population growth and continuous reproduction (Figure 5). These reproduction structures could depend on different seedling systems that were artificial mass seedling production in Lakes Jusan and Ogawara and natural seedling production in Lakes Shinji and Abashiri. All results obtained in this study allowed us to characterize the genetic diversity and reproduction structure in 4 brackish lakes as follows; continuous reproduction with a high diversity in Lake Shinji, rapidly grown reproduction with a low diversity in Lakes Jusan and Ogawara, continuous reproduction but irregular expansion with a low diversity in Lake Abashiri.

Passive dispersal of planktonic larvae may be strongly mediated by water movements, and the effect of water movements on the genetic diversity was commonly suggested in aquatic invertebrates [35] [36]. From spawning to Juveniles stage of *C. japonica* lasted approximately 12 days followed by planktonic stage to larval settlement for 54 hours to 6 days [37] [38]. Larvae reached the size of 4 - 5 mm shell length in 1 year and 14 - 15 mm in 3

**Table 5.** Mismatch distribution of *Corbicula japonica* collected in Lakes Shinji, Jusan, Ogawara and Abashiri.

Lake	Neutral test		Mismatch distribution			Goodness of fit test	
	Tajima's $D$	Fu's $F_s$	$\tau$	$\theta_0$	$\theta_1$	SSD	$H_{ri}$
Shinji	-0.2843	-1.4353	2.4473	0.0000	2.1085	0.0563	0.2071
Jusan	-2.2416**	-8.6093**	3.0000	0.0000	0.3849	0.0005	0.2893
Ogawara	-2.1064**	-8.6402**	0.3672	0.0000	99999.0000	0.0014	0.2351
Abashiri	-1.4684*	-3.2927**	3.0000	0.0000	0.2230	0.0136	0.5291

Parameters of the spatial expansion model and goodness of fit test to the model are shown with the respective significance for each clade. SSD, sum of squared deviations;  $H_{ri}$ , Harpending's raggedness index. \* $p < 0.05$ , \*\* $p < 0.01$ .



years, and those with >15 mm matured in Lakes Shinji [39], Ogawara [40] and Abashiri [41]. The spawning season of *C. japonica* in Lake Jusan was reported to be mid-July to late September [39], and that in Lake Ogawara was to be late July to early September [42]. It is noteworthy that *C. japonica* in Lake Abashiri failed to spawn from year to year due to low temperature and salinity [43]. The spawning season in Lake Shinji was, however, to be late March to early November and remarkably longer than that in the other lakes [42]. Iidzuka *et al.* [15] reported that the long spawning season might produce the complicated population genetic structure in Lake Shinji. It is therefore inferred that the difference of spawning in the season and length reflects in not only the genetic diversity but also the reproduction structure of *C. japonica* population in individual lake.

## 5. Conclusion

This study thoroughly compared population genetic structure of *C. japonica* among 4 major fishing brackish lakes in Japan by means of nucleotide sequence analysis of a 556 bp portion of the mtDNA COI gene. Although *C. japonica* is supposed to occur a recent expansion and form the current geographical distribution in Japan, its genetic diversity and reproduction structure has been developed specifically to the lakes examined in this study. In order not only to conserve *C. japonica* populations as a genetic resource but also to manage them as a fisheries stock, haphazard transplantation and import of seedlings and/or adults of *C. japonica* and its related species should be absolutely avoided.

## Acknowledgements

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