

Genetic Diversity and Evolution of Marine Animals Isolated in Marine Lakes

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1. Introduction

How do marine organisms genetically differentiate and speciate in illimitable oceans? It is considerably difficult to obtain clear answers to this question due to the following reasons. Marine organisms that reproduce by releasing numerous eggs and larvae are able to disperse over large distances and can therefore be distributed over large geographic areas. Such marine organisms have a large population size, gene flow between distant populations occurs frequently, and interspecies hybridization sometimes occurs (Kuriwa et al., 2007). Even geographically well-separated populations may be connected genetically, because there are few barriers to prevent gene flow in the oceans (Mayr, 1954; Palumbi, 1994). In contrast to the open ocean environment, the marine lakes of Palau (Western Caroline Islands), which are surrounded entirely by land and isolated from the sea, provide unique local environments for genetic differentiation of marine organisms (Dawson and Hamner, 2005; Gotoh et al., 2009; Goto et al., 2011). We have focused on marine lakes as isolated marine environments and have conducted continuous evolutionary studies of marine organisms in the Palau Islands for the past 13 years.

The Palau archipelago, which consists of volcanic islands to the north and a large number of elevated limestone islands to the south (U.S. Geological Survey, 1956; Hamner and Hamner, 1998), is surrounded by multiple hard-coral atolls. The adjacent waters of the Palau Islands are known as a hotspot with the highest level of marine species diversity (Allen, 2008). There are approximately 70 marine lakes on the limestone islands—commonly called the “Rock Islands”. It is thought that the marine lakes, which are small bodies of sea water in embayments and depressions on the limestone islands, have been gradually formed by floods resulting from rising sea levels after the Last Glacial Maximum (approx. 18,000 years ago); these waters were isolated from the surrounding barrier-reef lagoons and became sea-level marine lakes (Dawson and Hamner, 2005). Most of these lakes have been avoided by the local population due to the treacherous surface of the surrounding karst, lack of fresh water on the islands, and persistent myths that the marine lakes are haunted (Hamner and Hamner, 1998). Therefore, the ecosystems of most of the marine lakes have not yet been

disturbed by human activities. The fauna and flora in most of the marine lakes are quite different from those in the oligotrophic lagoons that have an abundance of hard corals; some diagnostic species of fish, mollusks, jellyfish, sponges, and green algae are observed in the lakes (Dawson and Hamner, 2005).

Thus, the marine lakes of Palau can be regarded as isolated and untouched marine environments, where the inhabitants confined to each lake are likely to develop into genetically distinct populations within a relatively small geographic range. Indeed, unique patterns of speciation and adaptation have been observed for jellyfish (Dawson and Hamner, 2005), sea anemone (Fautin and Fitt, 1991), foraminifera (Lipps and Langer, 1999; Kawagata et al., 2005), and bacteria (Venkateswaran et al., 1993) in the marine lakes; however, little is known about the evolutionary features of the nektonic and benthic animals in the lakes. Therefore, we focused on the fish and bivalve species in the marine lakes and analyzed their genetic diversity, differentiation, and population structures in comparison with those of related species, or different populations of the same species, that inhabit the barrier-reef lagoons.

In this chapter, we firstly introduce the general features of genetic diversity and evolution of the coral fish inhabiting the barrier-reef lagoons. Secondly, we discuss the genetic diversity and differentiation of marine lake populations of fish and bivalves inhabiting different marine lakes in comparison with those of the outer lagoons. Finally, we discuss genetic differentiation and speciation with respect to the marine lake model as an isolated marine environment.

2. Genetic diversity and evolution in coral reefs

Marine organisms that reproduce by releasing numerous eggs and planktonic larvae are able to disperse over considerable distances via large-scale currents; indeed, many adult-stage nektons frequently migrate across oceans. Therefore, marine organisms often show low levels of genetic differentiation even over large geographical ranges (Grant and Bowen, 1998), because ocean currents and/or the apparent lack of physical barriers to movement appear to facilitate extensive gene flow (Avice, 2000; Palumbi, 1994).

However, various groups of marine organisms have speciated and actually show a high level of species diversity, especially on tropical coral reefs. Here, we introduce the genetic diversity and multiple natural hybridizations of rabbitfish (Teleostei: Siganidae) distributed on tropical and subtropical coral reefs (Kuriwa et al., 2007).

Twenty-two rabbitfish species in the genus *Siganus*, described in the Western Pacific (Woodland, 1990; Randall and Kulbicki, 2005), are easily identified on the basis of species-specific coloration. We conducted phylogenetic analyses among 19 nominal *Siganus* species based on mitochondrial cytochrome b gene and nuclear ribosomal DNA internal spacer 1 (ITS1) sequences to infer their phylogenetic relationships and degree of genetic differentiation among species. We predicted that reproductive isolation is completely established among siganid species because teleost fish with opsin genes, such as RH1, RH-2, LWS, and SWS (Register et al., 1994; Yokoyama and Yokoyama, 1996; Seehausen et al., 2008), can visually discriminate individuals of the same species according to their coloration. As shown in Fig. 1, phylogenetic analyses using 2 different DNA markers indicated that most species were sufficiently genetically differentiated. However, we were surprised to

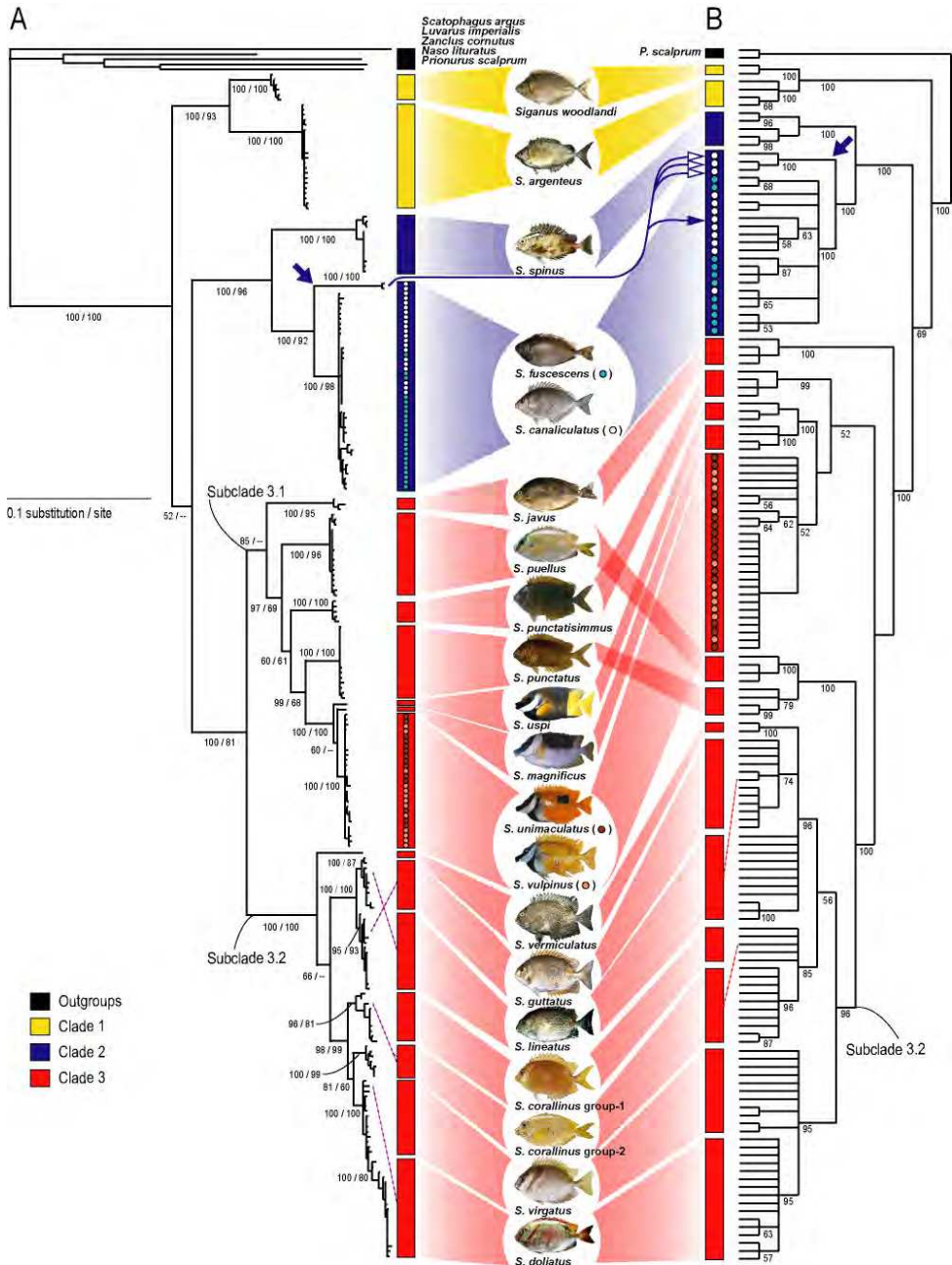


Fig. 1. Phylogenetic trees of siganid fish. (a) A 50% majority rule consensus tree derived from Bayesian analysis of mitochondrial (mt) cytochrome b sequences from 19 siganid species and 5 outgroup species with the GTR + I + Γ model. The topology was basically

identical to that from the neighbor-joining method. The numerals below the nodes indicate the posterior probabilities of the Bayesian trees (left) and bootstrap values (%) from 1,000 replicates in the neighbor-joining tree (right). (b) A strict consensus tree of 9712 trees resulting from maximum parsimony analysis of nuclear ribosomal DNA (internal spacer 1, ITS1) sequences from 19 siganid species and an outgroup species. The numerals below the nodes indicate the bootstrap values (%) from 100 replicates. The broken line indicates individuals with mtDNA or ITS1 sequences different from those expected from their morphology. The solid arrow indicates the Indian-clades of *S. canaliculatus* and the arrowheads indicate the ITS1 types found in individuals of the clade (open arrowheads, Indian-ITS1-types; solid arrowhead, Pacific-ITS1-type). See text for details. Discordance between the phenotypes and genotypes is shown by the broken lines and arrowheads (From K. Kuriwa et al., *Mol. Phylogenet. Evol.* 45:69-80, 2007).

find a large number of hybrids in 11 of the 20 Siganidae species (2 genetically differentiated species, groups 1 and 2, were included in *S. corallinus*).

The populations of *S. fuscescens*, with a small irregular-dotted skin pattern and found in temperate waters, and *S. canaliculatus*, with a large white-dotted skin pattern and found in the subtropical waters of the Western Pacific, are genetically mixed, indicating that these species should be included in a single biological species. Similarly, the populations of *S. unimaculatus*, with a large black spot, and *S. vulpinus*, without spots, are also genetically mixed, indicating that they should also be included in a single biological species. The analyses further suggest that introgression, probably caused by partial gene flow, occurred between the closely related species *S. guttatus* - *S. lineatus* and *S. virgatus* - *S. doliatus*.

On the other hand, phylogenetic analyses suggested that a morphologically unidentified individual is probably a hybrid between the distantly related species *S. corallinus* group 2 and *S. puellus*. However, comparison of ITS-1 sequences among *S. corallinus* group 2, *S. puellus*, and the hybrid showed that genetic recombination occurred in the hybrid in the region (Fig. 2). The data reveal the possibility that genome rearrangements occurred after hybridization between the pair of different species and further speciation has started. Little is known about why such frequent hybridizations occur in rabbitfish; however, it must be related to their sympatric distribution in coral reefs, overlapping spawning season among most species, and simultaneous spawning behavior in which numerous eggs and larvae are released. Such large scale hybridization must also be more prevalent in marine fish that inhabit coral reefs than previously assumed, and may have some relevance to their diversification.

		1111 11111111111111111111111111111111 2 2 222 3 4 44444444444444444444444444444444	
	23333333334444 8888888889999999990000 333334444444444355555555666666666 2 5 888 4 2 334444444444443555555566666677777777		
	981234567890123 12345678901234567890123 4567890123456789012345678901234567 1 3 678 6 0 8901234567890123456789012345678901234567		
<i>puellus</i> -type	TTGTCCCCCT----- GTCGCTGTGCGCGCGCGGC	GAAAAGACCTG-----GCTTTA T A TGG T - AG-----AGAGAAAACAAAAAAAGAAAA----	
clone-18	C-GTCCCCCCCCCC -----	GAAAAGACCTG-----GCTTTA T A TGG T - AG-----AGAGAAAACAAAAAAAGAAAA----	
<i>corallinus</i> -type	C-GTCCCCCCCCCC -----	AAAAGACCTGCTGCAGAAATGCTCGGTCCTT - C GGA C T TGTCCCAACCAAGAGAAA-CAAA-----GAAAAAAA	
<i>puellus</i> -type	TTGTCCCCCT----- GTCGCTGTGCGCGCGCGGC	GAAAAGACCTG-----GCTTTA T A TGG T - AG-----AGAGAAAACAAAAAAAGAAAA----	
clone-14	TTGTCCCCCT----- GTCGCTGTGCGCGCGC	AAAAGACCTGCTGCAGAAATGCTCGGTCCTT - A --- T - AG-----AGAGAAAACAAAAAAAGAAAA----	
<i>corallinus</i> -type	C-GTCCCCCCCCCC -----	AAAAGACCTGCTGCAGAAATGCTCGGTCCTT - C GGA C T TGTCCCAACCAAGAGAAA-CAAA-----GAAAAAAA	

Fig. 2. Alignment of two recombinant internal spacer 1 (ITS1) sequences from an unidentified individual with the consensus sequence of its putative parental species. The recombinants are shown in the middle of each sequence trio. The boxes enclose the regions where the recombinant is identical to one of the parents. Only the variable regions from the alignment are shown (From K. Kuriwa et al., *Mol. Phylogenet. Evol.* 45:69-80, 2007).

3. Genetic diversity and evolution in the marine lakes of the Palau Islands

The effect of geographical isolation on speciation has been studied in terrestrial and freshwater organisms (Chiba, 1998; Kliman et al., 2000; Caccone et al., 2001; Calsbeek and Smith, 2003; Meyer et al., 1990; Barluenga et al., 2006); however, little is known about what effect this may have on marine organisms. In this section, we introduce the general aspects of “the marine lakes as a model for isolated marine environments” in the Palau Islands and the fauna of the marine lakes, and show the peculiar evolution of some marine animals as inferred from their genetic population structures.

3.1 Geological and limnological aspects of the marine lakes of the Palau Islands

The Palau Islands are located at 7°30' N, 134°30' E in the Western Pacific (Fig. 3) and are composed of approximately 350 small islands (U. S. Geological Survey, 1956). The limestone islands, called the “Rock Islands”, are covered with thick tropical forest and are located in the central and southern parts of Palau (Fig. 3). These islands contain more than 70 marine lakes (Fig. 4) (Hamner and Hauri, 1981).

As shown in Fig. 5, it can be geologically inferred that the marine lakes were gradually formed through the following steps. Firstly, the limestone derived from hard corals was shaped and uplifted during the Miocene and Pleistocene periods. Secondly, the islands were eroded by rain and wind, and numerous depressions were formed. Finally, with the rising sea level of the end of the Last Glacial Maximum, seawater flooded the depressions through fissures or tunnels (Hamner and Hauri, 1981; Hamner and Hamner, 1998). Geologically, the marine lakes were estimated to have formed in chronological series, namely, the deeper depressions flooded first (~12,000 years ago) and the shallower depressions flooded later (~5,000 years ago) (Dawson and Hamner, 2005).

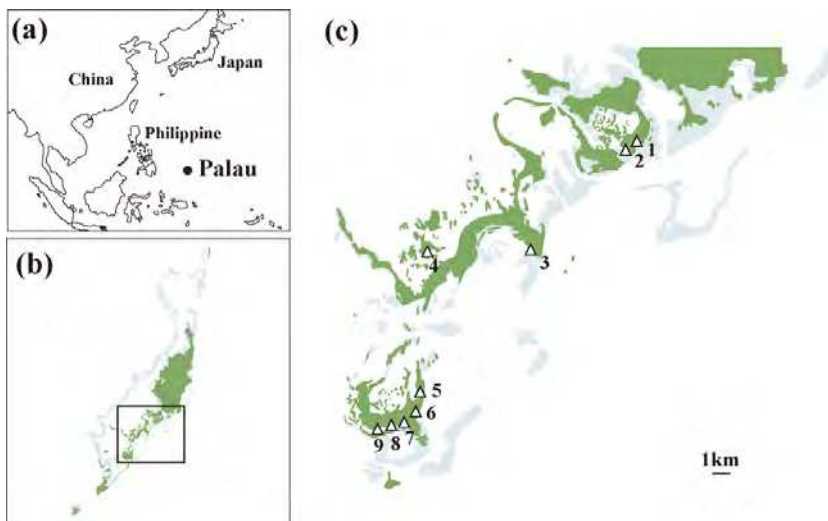


Fig. 3. Location of the Republic of Palau (a) and the surveyed major marine lakes 1–9 in (c) of the Palau Islands (b). The green and light blue portions indicate the islands and coral reefs, respectively.

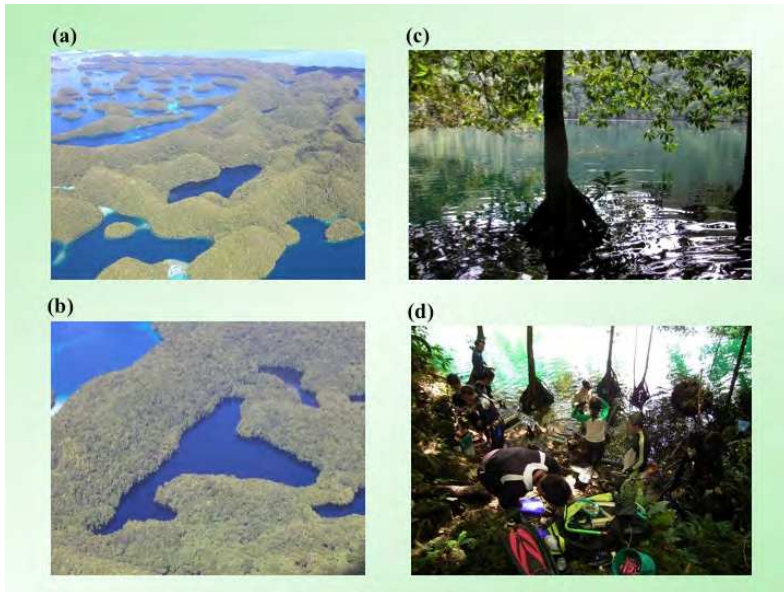


Fig. 4. (a), (b): Major marine lakes in the Palau Islands; (c), (d): Their shores covered with mangroves.

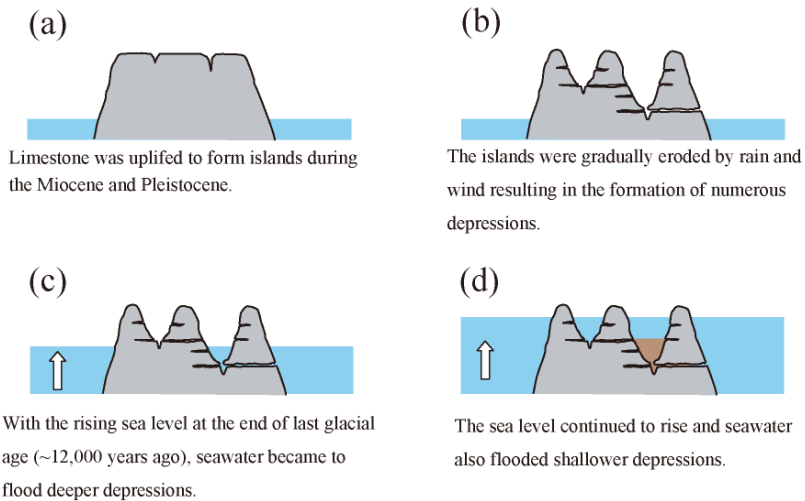


Fig. 5. (a) – (d). Geologically inferred formation process of the marine lakes. The brown portion indicates an anaerobic layer.

We surveyed more than 25 marine lakes in Palau and show the limnological characteristics of the 9 major marine lakes in Table 1. The major marine lakes of the Palau Islands are limnologically holomictic or meromictic lakes. As shown in Fig. 6, the holomictic marine lakes have retained hydraulic connections to the surrounding lagoons either at the surface

or through submarine tunnels and fissures in the fenestrated karst (Hamner et al., 1982), and water exchange and circulation regularly occur by tidal movement. On the other hand, the meromictic marine lakes have no apparent connection to the lagoons (Fig. 6). There are fewer tunnels or fissures where large organisms can pass through, although water exchange occurs through fissures and micropores in the limestone. In the meromictic lakes, thorough water-circulation does not occur because mixing by wind is reduced by the jungle-covered karst ridges. Topographic protection from wind, heavy regular rain throughout the year with precipitation exceeding evaporation, and modest tidal exchange produce stratified brackish waters above permanently anoxic saline hypolimnia (Hamner and Hamner, 1998). Mangrove forests develop on their coasts, and some fish species (e.g., *Apogonidae* spp., *Atherinidae* spp., and *Gobiidae* spp.), jellyfish (e.g., *Aurelia* spp. and *Mastigias* spp.), mussels (*Brachidontes* spp.), many sponges (e.g., *Haliclona* spp.), and sea anemones (e.g., *Entacmaea medusivora*) live in these marine lakes. Even if there were tunnels or fissures in deeper areas, living organisms from the open water could not have colonized the lakes because the deep anoxic layers, which include fatal chemicals such as hydrogen sulfide, would prevent their migration. Therefore, the organisms inhabiting meromictic lakes are thought to have been isolated since the lakes were formed and to have evolved endemically in each lake.

Site	Abbreviation	Depth (m)	Length (m)		Physical structure
			Long axis	Width	
1 Uet era Ngerumeuangel, Koror Is.	NLK	38	270	210	Meromictic
2 Goby Lake, Koror Is.	GLK	15	195	110	Meromictic
3 Shrimp Lake, Ngerktabel Is.	SHN	5	100	55	Meromictic
4 Uet era Ongael, Ongael Is.	OLO	4	150	100	Holomictic
5 North Cassiopea Lake, Mecherchar Is.	NCM	4	135	55	Holomictic
6 Jellyfish Lake, Mecherchar Is.	JFM	32	420	150	Meromictic
7 Big crocodile Lake, Mecherchar Is.	BCM	22	575	290	Meromictic
8 Spooky Lake, Mecherchar Is.	SPM	14	230	65	Meromictic
9 Clear Lake, Mecherchar Is.	CLM	30	320	225	Meromictic

Table 1. Limnological characteristics of the surveyed major marine lakes. Site Nos. 1-9 correspond to those shown in Fig. 3 (c).

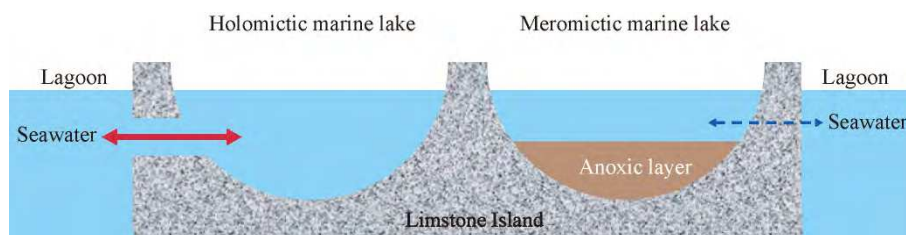


Fig. 6. Schematic figures of the meromictic and holomictic marine lakes in the Palau Islands.

3.2 Fauna in the marine lakes

As mentioned above, species diversity is extremely high in the Palau Islands and numerous fish and benthic animal species inhabit the coral reefs. On the other hand, unique fauna, which is quite different from that of the coral reefs, is maintained in the marine lakes. As shown in Table 2, restricted fish, bivalves, and jellyfish species inhabit the marine lakes and

these marine lake-specific diagnostic species are not distributed in all of the marine lakes. The numbers of individuals are extremely high in whichever lakes the species inhabit. In a recent report on copepods, we demonstrated that the species diversity in the marine lakes is clearly lower than in the open sea around the Palau Islands, particularly for copepod species preferring to low-salinity waters, such as *Oithona dissimilis* and *Bestiolina similis*, that inhabit the marine lakes (Saitoh et al., 2011).

Species	Korol Is.		Ngerktabel Is.	Ongael Is.	Mecherchar Is.				
	NLK	GLK	SHL	ONG	NCM	JFM	BCM	SPM	CLM
Cnidaria									
<i>Aurelia</i> sp.4		++		(++)		(++)	++		
<i>Mastigias</i> spp.	++	++		(++)		(++)			++
<i>Cassiopea</i> sp.			+	(++)	+				
Mollusca									
<i>Brachidontes</i> spp.	++	++	++	++	++	++	++	++	++
Chordata									
<i>Atherinomorus endrachtensis</i>				++	++	++	++	+	+
<i>Sphaeramia orbicularis</i>	++				+	++			++
<i>Apogon lateralis</i>				++	++		++	++	++
<i>Acentrogobius janthinopterus</i>	++	++	++			++	++	++	++
<i>Exyrias puntang</i>	++	++		++	++				

++ numerous; + moderate; () indicates large scale fluctuation in the number of individuals

Table 2. Dominant species and their population size in the marine lakes of Palau.

A moon jellyfish, *Aurelia* sp. 4 (Dawson and Jacobs, 2001) only inhabits the marine lakes, while different biological species, *Aurelia* sp. 3 and *Aurelia* sp. 6, only inhabit the surrounding lagoons. Dawson (2005) also described 5 new subspecies of *Mastigias* jellyfish distributed in different marine lakes of Palau. The subspecies are morphologically different, particularly for the shape of their oral arms, from *Mastigias papua*, which is distributed in the open sea of the Pacific. We have also confirmed that each of the 5 subspecies is slightly morphologically different from each other, whereas the morphology of 4 subspecies, except *M. papua etpisoni*, has changed every year. Thus, the 5 subspecies of *Mastigias* jellyfish have been geographically isolated in each marine lake and have evolved, whereas the 4 subspecies are pleiotropic.

Mussels, *Brachidontes* spp., are also only observed in the marine lakes located on different islands. A cardinal fish, *Apogon* sp., is also probably only distributed in the marine lakes. Numerous gobies, such as *Acentrogobius janthinopterus* and *Exyrias puntang*, inhabit the marine lakes, although small numbers of individuals are occasionally observed in the lower reaches of rivers flowing to the lagoons. Several species of sponges (e.g., *Haliclona* spp.), sea anemones (e.g., *Entacmaea medusivora*), sea cucumbers, gastropods, and simple and colonial ascidians are also only found in the marine lakes.

On the other hand, numerous individuals of a cardinal fish, *Sphaeramia orbicularis*, and a silverside, *Atherinomorus endrachtensis*, are found in the marine lakes and outer lagoons.

Furthermore, we found distinctive phenomena indicating that the number of individuals of jellyfish widely fluctuated. First we observed massive deaths of *Aurelia* and *Mastigias* spp. in Jellyfish Lake, Mecherchar Island. A large scale El Niño-Southern Oscillation (ENSO)

occurred during 1997–1998 around Palau, resulting in dry weather without any rain for 6 months, an abnormal rise in the seawater temperature, and the extinction of many hard corals and other animals dependent on the corals (personal communication from Carp Corporation staff). We could not observe any adults of *Aurelia* and *Mastigias* spp. in Jellyfish Lake during our expedition at the beginning of 1999. However, we could find some adults of both species during our expedition at the end of 1999, and the number of adults had explosively increased within a few years after the ENSO.

We also observed similar wide fluctuations in the number of individuals in Ongael Lake. This lake is shallower (~4 m deep) than the other marine lakes and its environmental conditions must, therefore, be more unstable. Three jellyfish species of the genera *Aurelia*, *Mastigias*, and *Cassiopea* inhabit this lake; however, the number of adult individuals of each species has drastically fluctuated on a cycle of a few years. Namely, adult individuals of any species continue to increase in number for a few years, and suddenly become extinct. But, the number of adults suddenly begins to increase some years later. Thus, fluctuations in the number of adults are common in jellyfish populations. However, the habitats and dynamics of their polyps are unknown. If we can survey them, we may be able to analyze how such short term fluctuations affect the genetic diversity in their populations.

3.3 Genetic diversity and evolution in the marine lake populations

3.3.1 Genetic population structures and divergence of a cardinal fish, *S. orbicularis*

S. orbicularis inhabits meromictic marine lakes and lagoons (Table 3). We determined the complete sequence of the mitochondrial control region (CR: 824 bps) and compared the genetic structure of marine lake and lagoon populations (Gotoh et al., 2009).

CR polymorphisms in *S. orbicularis* A total of 17 haplotypes were detected from 157 individuals collected from 3 meromictic marine lakes and 3 lagoon sites. The base substitutions included 18 transitions and one indel. The base composition of the different haplotypes were 29.4–29.7% for A, 29.9–30.1% for T, 16.9–17.2% for G and 23.2–23.5% for C, and no remarkable deviation in the composition was observed among haplotypes. As for many other teleost fishes, most substitutions were observed in the 5' terminal side, which is known to be a hyper variable region (Lee et al., 1995). Only 5 of all haplotypes were shared among different populations (Table 3). The haplotype So01 that has the highest frequency among lagoon populations was detected in two individuals from the JFM lake, while none of the other haplotypes were shared between lagoon and marine lake populations.

Genetic differentiation among the marine lake populations in *S. orbicularis* The haplotype diversity (h) and the nucleotide diversity (π) are shown in Table 4. In marine lake populations, the values ranged from 0.067 to 0.465 for h and from 0.008 to 0.113 for π . In lagoon populations, these values ranged from 0.423 to 0.815 and from 0.103 to 0.198, respectively. In pooled lagoon samples, these values were 0.618 and 0.162, respectively.

Lagoon populations share some haplotypes (Table 3). The AMOVA without group design shows a high percentage of variation within populations (90.20%: Table 5) and a significant pairwise Φ_{ST} of 0.098 ($P < 1e-03$). The AMOVA with two groups calculated with SAMOVA does not show a significant value of F_{CT} (0.159, $P = 0.348$; Table 5).

Haplotype	Position of differences												Sampling sites and frequency						Accession No.					
	3	31	37	132	173	194	221	332	359	478	510	530	568	570	664	Marine lake				Lagoon				
																NLK	CLM	JFM		PPE	JFOS	CMR		
So01	C	T	C	G	C	A	G	C	G	A	A	A	G	A	-	2	18	23	1	1	19	8	AB252837	
So02	G	A	G	.	18	29	7	1	1	19	8	AB252838	
So03	A	A	G	.	18	29	7	1	1	19	8	AB252839	
So04	G	A	G	.	23	23	7	1	1	19	8	AB252840	
So05	G	A	T	G	.	7	7	7	1	1	19	8	AB252841	
So06	G	A	G	.	1	1	7	1	1	19	8	AB252842	
So07	A	A	.	A	G	.	.	.	G	.	1	1	1	1	1	19	8	AB252843	
So08	A	A	.	A	G	.	1	1	1	1	1	19	8	AB252844	
So09	A	A	.	A	.	G	.	.	G	.	1	1	1	1	1	19	8	AB252845	
So10	A	G	.	2	2	2	2	2	2	2	8	AB252846
So11	G	.	1	1	1	1	1	1	1	17	AB252847
So12	T	G	.	1	1	1	1	1	1	1	17	AB252848
So13	A	A	G	.	1	1	1	1	1	1	1	17	AB252849
So14	G	A	G	C	1	1	1	1	1	1	1	17	AB252850
So15	G	.	1	1	1	1	1	1	1	17	AB252851
So16	.	C	G	.	1	1	1	1	1	1	1	17	AB252852
So17	G	.	1	1	1	1	1	1	1	17	AB252853

Table 3. Variable sites of the 17 haplotypes and number of individuals for each haplotype by sampling area. Abbreviations of marine lakes are common with Tabales 1 and 2. PPE: a part of Peleliu Island; JFOS: outside of Jellyfish Lake; CMR: mouth of Comet River (Modified from R. O. Gotoh et al., *Genes Genet. Syst.* 84:287-295, 2009)

Location	Sample size n	H	h	π (%)	Tajima's D		Goodness-of-fit test	
					D	P	SSD	P
Marine lakes								
NLK	26	3	0.465±0.086	0.113±0.089	0.447	0.689	0.174	0.100
CLM	30	2	0.067±0.061	0.008±0.019	-1.147	0.038*	0.000	0.250
JFM	26	3	0.219±0.103	0.063±0.061	-1.071	0.180	0.027	0.150
Lagoon								
PPE	26	8	0.526±0.118	0.147±0.108	-0.684	0.293	0.383	0.000*
JFOS	25	5	0.423±0.119	0.103±0.084	-0.543	0.338	0.019	0.550
CMR	24	6	0.815±0.044	0.198±0.135	0.639	0.763	0.020	0.100
LAG ^a	75	10	0.618±0.060	0.162±0.113	-0.501	0.330	0.035	0.180

H: number of haplotypes; h: haplotype diversity; π : nucleotide diversity; D: Tajima's D value; SSD: sum of squared deviations.

*: $P < 0.05$.

^a: LAG consists of pooled samples of all lagoon individuals.

Table 4. Control region sequence diversity, Tajima's D and goodness-of-fit tests (Modified from R. O. Gotoh et al., *Genes Genet. Syst.* 84:287-295, 2009).

The values of pairwise Φ_{ST} ranged from 0.429 to 0.870 among marine lake populations, from -0.008 to 0.181 among lagoon populations, and from 0.531 to 0.848 between marine lake populations and lagoon populations. Almost all of these values were significant at a level of 0.05% (Table 6).

Structure tested	Source of variation	Observed partition		P
		% total	Φ statistics	
1. One gene pool	Among populations	9.8	$\Phi_{ST} = 0.098$	0.000
	Within populations	90.2		
2. Two gene pool (PPE, JFOS)(CMR) ^a	Among groups	15.9	$\Phi_{CT} = 0.159$	0.348
	Among populations	-1.1	$\Phi_{SC} = -0.013$	0.000
	Within populations	85.2	$\Phi_{ST} = 0.1479$	0.000

^a By maximizing Φ_{CT}

Table 5. Multiple hierarchical analyses of control region in samples of *S. orbicularis* (From R. O. Gotoh et al., *Genes Genet. Syst.* 84:287-295, 2009).

	NLK	CLM	JFM	PPE	JFOS	CMR
NLK		0.000	0.000	0.000	0.000	0.000
CLM	0.844**		0.000	0.000	0.000	0.000
JFM	0.429**	0.870**		0.000	0.000	0.000
PPE	0.713**	0.781**	0.669**		0.448	0.014
JFOS	0.763**	0.848**	0.740**	-0.008		0.002
CMR	0.612**	0.659**	0.531**	0.094*	0.181*	

* $P < 0.05$, ** $P < 0.001$

Table 6. Population pairwise Φ_{ST} values for control region (below the diagonal) and P values (above the diagonal) (Modified from R. O. Gotoh et al., *Genes Genet. Syst.* 84:287-295, 2009).

Phylogenetic relationships among haplotypes in *S. orbicularis* We constructed the statistical parsimony network as shown in Fig. 7. For the CLM population, haplotype So07 was derived from the high frequency haplotype So03. In the JFM population, the major haplotype So04 connected to haplotype So14 through one indel and with haplotype So01, which was shared with lagoon populations, through three substitutions. All the haplotypes detected from the NLK population were derived from haplotype So04. Finally, haplotypes recognized in lagoon populations connected to each other through one to four substitutions.

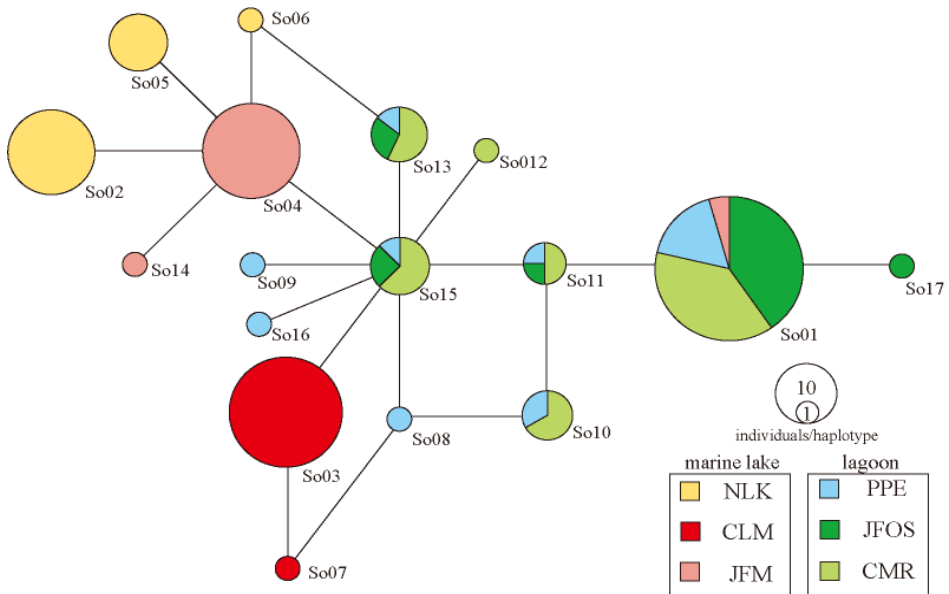


Fig. 7. Statistical parsimony network for haplotypes detected in marine lake and lagoon populations of *S. orbicularis*. Size of circles represents the frequency of each haplotype. Letters beside the circles indicate each labeled haplotype (Modified from R. O. Gotoh et al., *Genes Genet. Syst.* 84:287-295, 2009).

Neutrality and demographic history in populations of *S. orbicularis* Results of Tajima's D and goodness-of-fit tests are shown in Table 4. The values of Tajima's D test were not significant except for the CLM population. In the goodness-of-fit test, only the value of the PPE population was significant. In the mismatch distribution analysis (Fig. 8), the CLM and JFM populations showed similar results where the peak of frequency distribution in the number of nucleotide substitution is near zero. The NLK population showed two distinctive peaks. The pooled lagoon population showed a similar pattern with a slightly moderate curve compared with marine lake populations.

Historical changes of lagoon populations in *S. orbicularis* The results of the AMOVA could not sufficiently clarify the genetic structure of the lagoon populations. However, the pairwise Φ_{ST} values between CMR and PPE, and CMR and JFOS indicate that some genetic divergence has occurred. This divergence is likely due to mouth breeding of *S. orbicularis*, a particular behavior well known among apogonids. In these species, males brood the eggs in their mouth for approximately eight days before releasing larvae (Myers, 1999). After a short

pelagic stage, the juveniles settle down in dark habitats among mangrove roots, limestone rocks, or in shallow piers along the shoreline. Therefore, large-scale dispersal by tidal currents is not likely to occur widely. Indeed, previous studies on two fish species, Banggai cardinalfish, *Pterapogon kauderni* and black surfperch, *Embiotoca jacksoni*, lacking a pelagic larval phase revealed low levels of gene flow and strong phylogeographic breaks within their 100 km to 1,000 km geographic ranges (Bernardi and Vagelli, 2004; Bernardi, 2000).

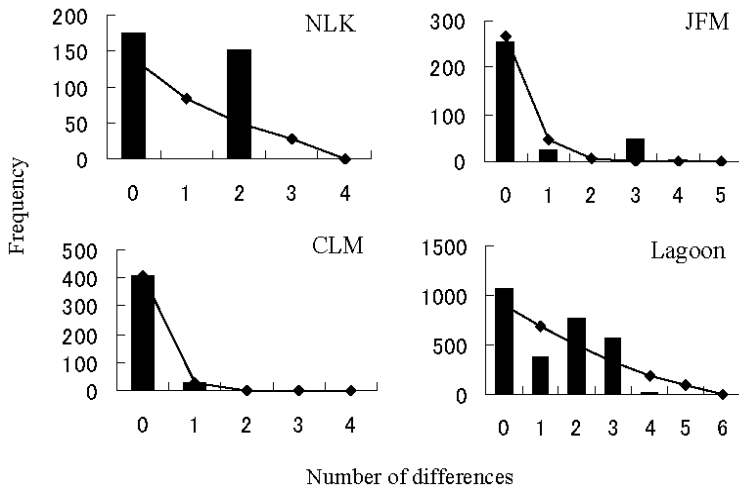


Fig. 8. Mismatch distribution analysis based on haplotypes of control region in each population of *S. orbicularis*. The vertical bars are the observed distribution of mismatches and the line represents the expected distribution under the sudden-expansion model (Modified from R. O. Gotoh et al., *Genes Genet. Syst.* 84:287-295, 2009).

Lagoon populations showed high haplotype and low nucleotide diversities. Grant and Bowen (1998) have analyzed the genetic diversity in populations of sardines and anchovies based on mtDNA sequences and classified them into four patterns. According to their classification, the lagoon populations in *S. orbicularis* correspond to Category 2, indicating that the population size increased and genetic variations have accumulated after its decrease. In mismatch analysis, bottlenecks yield either a bimodal distribution or a distribution close to zero, depending on whether the bottleneck reduced or completely removed the genetic diversity (Frankham et al., 2002). The mismatch analysis performed here suggests that the lagoon populations of *S. orbicularis* have experienced size fluctuations. This sudden expansion model was further supported by the goodness-of-fit test ($P_{ssd} = 0.18$).

Two historical scenarios are considered as the factors led to these results. The first is that the recent colonization of a small number of individuals in Palau islands (founder event), and afterwards their population size increased with low level of migration with different haplotypes. The migration of individuals having different haplotypes is rarely occurred after the establishment of lagoon population, because *S. orbicularis* is not likely to extensive disperse, as described above. The second is that the reduction of population size caused by large-scale climate fluctuations (bottleneck event). Fauvelot et al. (2003) compared the

genetic structure of several Polynesian species of coral fish populations inhabiting outer and inner lagoons, and reported that the genetic diversity in the inner lagoons was lower than that in the outer ones. From these results, they further speculated that the low level of genetic diversity found in the inner lagoons was due to a strong bottleneck that occurred through a drought derived from the lower sea level in inner lagoons during the Ice Age. Approximately 10,000 years ago, the sea level on the Palau Islands was 20 meters lower than at present (Kayanne et al., 2002). It is thus plausible that the population size and genetic diversity of *S. orbicularis* lagoon populations decreased with the lower sea level and then rapidly increased after the bottleneck event so that mutations could accumulate in the populations.

Historical changes of marine lake populations in *S. orbicularis* In contrast to lagoon populations, marine lake populations show low haplotype and nucleotide diversities (Table 4). Although the level of genetic diversity in the NLK population was close to that in the lagoon populations, the NLK population possessed less haplotypes, similar to the other marine lake populations (Table 3). Therefore, we regard this lower genetic diversity as a common conspicuous feature for all types of marine lake.

All marine lake populations indicating low haplotype and nucleotide diversities correspond to Category 1 of Grant & Bowen (1998) classification. This category suggests that a population has experienced founder and/or bottleneck events during the last thousand to tens of thousand years. Moreover, results for the mismatch distribution and Tajima's D value for JFM and CLM populations reveal that their population sizes have increased after a decrease (Tajima's D: CLM D = -1.147, P = 0.038; JFM D = -1.071, P = 0.180). The sudden expansion model was further supported for each population (goodness-of-fit test: CLM Pssd = 0.25; JFM Pssd = 0.15).

The founder events when the marine lakes were formed must have affected the present genetic diversity of marine lake populations. Furthermore, large-scale climate fluctuations, such as the El Niño-Southern Oscillation (ENSO) also possibly affected their population size decrease (bottleneck event). The strongest ENSO actually took place in 1997 - 1998 (McPhaden, 1999) and the precipitations on the Yap Islands (located 100 km north of the Palau Islands) were much lower than those of an ordinary year (Kimura et al., 2001). Furthermore, it did not rain for half-a-year on the Palau Islands and the surface temperature of the outer sea rose by 1 to 4 °C, resulting in a large salinity increase (Iijima et al., 2005). It was reported that a large number of marine organisms such as corals and jellyfishes died at the time (Bruno et al., 2001). We also observed that adults of golden and moon jellyfishes inhabiting the JFM lake disappeared in 1999. Because ENSO has taken place every 10 to 15 years (Iijima et al., 2005), such environmental changes must have occurred repeatedly since the marine lakes were formed approximately 10,000 years ago. It is likely that the marine lake species have frequently suffered the heavy bottleneck effect under the short intervals of ENSO and kept low level of genetic diversity in the populations. The number of individuals might have rapidly increased under the stable climate, as we actually observe in jellyfish species, because the marine lake species can reproduce frequently in a year and grow in the tropical eutrophic marine lakes.

It is also possible that the reproduction and the survival of *S. orbicularis* isolated in marine lakes have been affected by ENSO and that their population size decreased, although we could not estimate their decrease exactly at the time. Because genetic drifts have strong

impacts on small populations, the level of genetic diversity of marine lake populations decreased to very low levels and then genetic structures changed. Ultimately, each marine lake population developed a distinctive genetic structure (pairwise Φ_{ST} among marine lake populations range from 0.429 to 0.870) (Table 6).

The NLK population shows a slightly different network pattern (Fig. 7), where two major and one minor haplotypes were detected. The effect of increased genetic drift after the founder and the bottleneck events usually leads to the fixation of a single haplotype. However, it is probable that a few haplotypes have been maintained in a population when the population size exponentially increased before the haplotype fixation and then has been stable. We hypothesized that the different pattern between the NLK and the other marine lake populations may be related with areas and depth to anoxic layer of each marine lake because the population size and its stability are dependent on such spatial factors. However, the areas and depth of the marine lakes we studied are very similar (area: JFM = 50,000 m²; NLK = 43,000 m²; CLM = 39,000 m², depth to anoxic layer: JFM = 15 m; NLK = 10 m; CLM = 15 m; Dawson & Hamner, 2005; Hamner & Hamner, 1998). In this study, we cannot conclude why only the NLK population demonstrates the different pattern and whether the pattern was caused by chance or not. To address these questions, we will need to survey more detailed information about each marine lake and conduct population analyses by using nuclear DNA markers.

Evolutionary features of marine lake populations in *S. orbicularis* There was no common haplotype among marine lake populations and all haplotypes were unique to each marine lake except haplotype So01 observed in the JFM lake (Fig. 7). We consider that haplotype So01 did not occur in the JFM lake independently, because haplotype So01 is distant from the major haplotype So04 in the JFM population (Fig. 7). Although two fissures were found in this lake (Hamner & Hamner, 1998), there is no observation that marine organisms moved in or out through these fissures. We think the following two possibilities about this finding. The first is that the haplotype So01 originally possessed in the founder population has remained through severe genetic drifts. The second is that the individuals having the haplotype So01 have been artificially introduced from the lagoon. Many tourists visit the Jellyfish Lake because it is a well-known snorkeling spot where they can swim with numerous jellyfishes and observe them. In fact, the non-indigenous, invasive sea anemone *Aiptasia* sp. and the sponge *Haliciona* sp. were first observed at the foot of the dock where visitors enter the lake and then extend their distribution (Marino et al., 2008). However, we have no measure to confirm which is true for the present.

Pairwise Φ_{ST} among marine lake and lagoon populations ranged from 0.531 to 0.848 (Table 6), indicating a high genetic differentiation between these populations. The peripatric differentiation between marine lake and lagoon populations was caused by a small number of individuals colonizing the lakes from the lagoons (founder event) followed by repetitive bottleneck events, such as those generated by ENSO. So far, such higher genetic divergences in extremely short geographical ranges (approximately 150 - 250 m) have scarcely been reported for marine organisms. The marine lake is thus a model that could clarify the process of evolution by geographical isolation in these organisms.

It is well known that approximately 300 species of cichlids have rapidly speciated in Lake Victoria, Africa, during the last 12,000 years (Johnson et al., 1996). Although most of the species are not divergent both in mitochondrial and nuclear gene sequences, they are morphologically

and ethologically differentiated and reproductive isolation is established (Meyer et al., 1990; Verheyen et al., 2003; Nagl et al., 1998; Terai et al., 2004). In a preliminary study, we compared some morphological characters, such as the number of spinous and soft fin ray counts and the scalation among populations in *S. orbicularis* but apparent differences were not found (data not shown). However, we recognized that feeding and escaping behaviors are apparently different between marine lake and lagoon populations. Because individuals in marine lakes bait actively, sometimes even feeding on wounded individual of the same species, and seldom escape without wariness, we can easily collect them by angling. Conversely, individuals in lagoons always hide carefully in dark places, and often escape quickly, making them very difficult to collect. Such behavioral difference between marine lake and lagoon populations may be due to a difference in the number of predators (such as carnivorous fishes) between these two habitats, the lakes having fewer predators than the lagoons. We will further survey quantitative characters such as the length of the caudal fin and the body depth in the future study, because these characters are possibly influenced by behavior. Vamosi (2003) has reported that a change in predation pressure could have promoted speciation in the threespine stickleback *Gasterosteus aculeatus* where a release from predation pressure would have led them to speciation. Such ecological factors may also promote rapid adaptive evolution in completely isolated marine lake populations of *S. orbicularis*.

3.3.2 Genetic population structures and speciation of mussels, *Brachidontes* spp.

Marine lake mussels inhabit many meromictic marine lakes, although we have been never found in holomictic marine lakes and lagoons. We collected the mussels from 9 meromictic marine lakes in Palau and conducted morphological, phylogenetic and population genetic characterization (Goto et al., 2011).

Morphological differences between three morphs of marine lake mussels General morphological characters were similar among the marine lake mussels, and agreed with those of the family Mytilidae. We further found some morphological differences in the maximum shell length, thickness, ratio of shell height to width (height/width ratio), color and clearness of radiating ribs among individuals inhabiting different marine lakes. According to these differences, the mussels were sorted into three morphs: NS, ON and MC (Fig. 9). Only a single morph was found in each marine lake.

Phylogenetic position of the three morphs of mussels We conducted phylogenetic analyses of 3 morphs of mussels based on 18S ribosomal (r) RNA genes to infer their phylogenetic positions. We found no sequence variation in the 18S rRNA genes among the individuals. The phylogenetic trees (Bayesian, ML and MP trees) constructed from the data set consisting of the marine lake mussels and other genera in Mytilidae generally agreed well with each other (Fig. 10; ML and MP trees are not shown). The clade consisting of *Brachidontes* and *Hormomya* species (*Brachidontes*-*Hormomya* complex) was supported by high posterior probabilities for Bayesian analysis (1.00) and high bootstrap values for ML analysis (100%). The trees clearly indicated that the marine lake mussels are positioned in a single clade within the *Brachidontes*-*Hormomya* complex. Of the species included in our analysis, the marine lake mussels were most closely related to *H. mutabilis* collected from Okinawa, Japan. The phylogenetic trees also suggested that *G. demissa* is the sister taxon to the complex, although this was not supported statistically. The *Brachidontes*-*Hormomya* complex and the marine lake mussels formed an unresolved polytomy in these analyses.

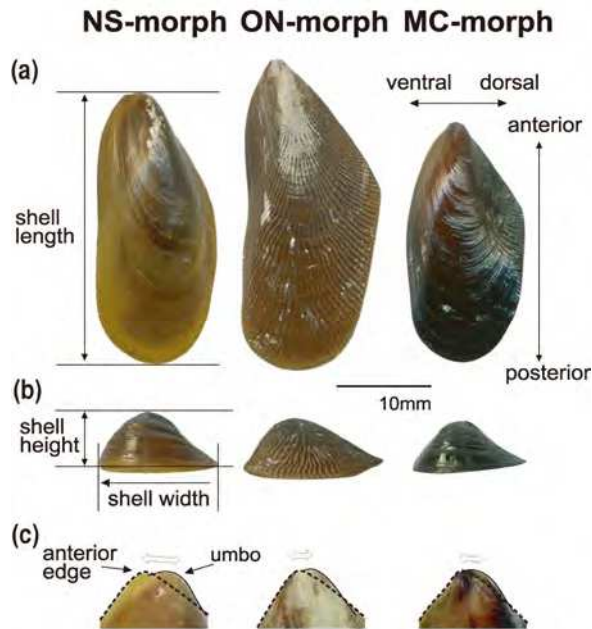


Fig. 9. Three morphs of marine lake mussels. (a) The surface of left valves. Strong divaricate radial ribs are observed over the whole surface of the ON-morph. Weak ribs are present on the whole surface of the NS-morph and dorsal sides of the MC-morph. (b) Left valves viewed from the rear. The ratio of shell height to width of the NS-morph is higher than that of the other morphs. (c) Enlargements of the anterior edge. The umbo of the NS-morph is relatively distant from the anterior edge, whereas those of the ON- and MC-morphs are close to the anterior edges (From T. V. Goto et al., *Zool. Sci.* 28:568-579, 2011).

Phylogenetic relationships among three morphs of marine lake mussels To infer phylogenetic relationships among three morphs of marine lake mussels, we further detected their mitochondrial cytochrome c oxidase subunit 1 (CO I) gene sequences and carried out phylogenetic and population genetic analyses. However, mussels have a unique mode of mitochondrial DNA (mtDNA) inheritance: doubly uniparental inheritance (DUI) (Zouros et al., 1994a, b) or gender-associated inheritance (Skibinski et al., 1994a, b) that two types of mitochondrial genome, the Female (F)- and Male (M)-types, are transmitted to the offspring. Females transmit F-type mtDNA to both female and male offspring through the eggs, whereas males only transmit M-type mtDNA to male offspring through the sperm. In adult males, F-type mtDNA prevails in the somatic tissue, while M-type mtDNA occurs predominantly in the gonads (Stewart et al., 1995; Sutherland et al., 1998). The F- and M-types of mtDNA evolved independently and two highly diverged mitochondrial genomes are retained in all male individuals. F-type mtDNA is more easily used as a genetic marker to study the phylogeny of mussels than M-type mtDNA, because it exists in both sexes, and reference sequences for *Brachidontes* mussels are available (Lee and Foighil, 2004, 2005; Terranova et al., 2007; Samadi et al., 2007). Therefore, in the present study, maternal and paternal CO I gene sequences were first distinguished, and then the F-type CO I gene was used for the phylogenetic analysis.

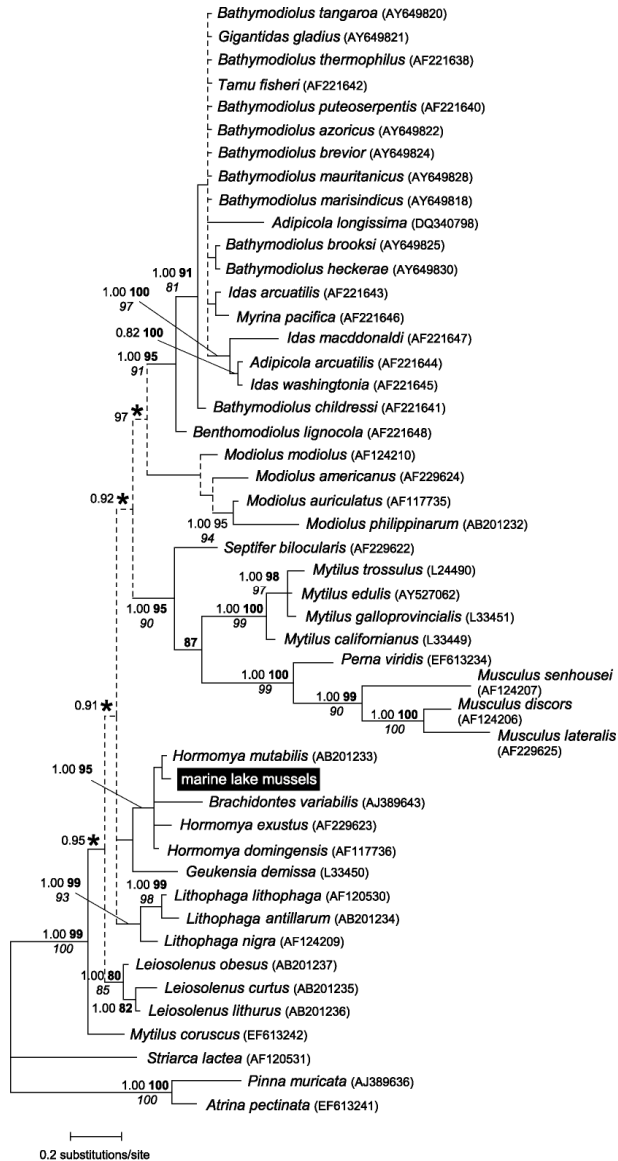


Fig. 10. 18S rRNA gene-based Bayesian tree showing the phylogenetic position of marine lake mussels in the family Mytilidae. The inferred clades of the Bayesian, ML and MP trees (solid line) agreed with each other, but partial topologies (broken line) were not in agreement among the three trees. Asterisks indicate clades of the Bayesian and ML trees that agreed with each other. Bayesian clade posterior probabilities (>0.80 , normal) are indicated with ML (>80 , italic); bootstrap support values at each branch. The sequence of *Brachidontes exustus* was registered as *Hormomya exustus* in the DNA databases and the registered name was used in this phylogenetic tree (From T. V. Goto et al., *Zool. Sci.* 28:568-579, 2011).

We analyzed F-type COI gene sequences together with M-type COI gene sequences obtained from three specimens to further determine the phylogenetic relationship between the three morphs. No indels were found in 555 bp of the F- and M-type COI gene sequences. A total of 19 haplotypes were detected from the F-type COI genes of the three morphs, and none of these were shared between the different morphs. We found two M-type haplotypes from three ON-morph specimens collected in OLO. The average genetic distance between the F- and M-type sequences was 0.186 (p-distance); the level of genetic divergence was close to that reported among *Mytilus* species (Mizi et al., 2005).

The phylogenetic relationships between the three morphs and 14 reference sequences retrieved from DDBJ were inferred using Bayesian, ML and MP approaches for these F- and M-type COI gene sequences (only the Bayesian tree is shown in Fig. 11). The F-type COI gene sequences of the marine lake mussels, including 96 polymorphic sites with 75 transitions and 25 transversions, were clustered into two distinct lineages, A and B, with an average genetic distance of 0.149 (p-distance). Monophyly of each of the lineages A and B was strongly supported by posterior probabilities (0.99 and 1.00, respectively) and bootstrap values (lineage A, 95% in ML and 100% in MP; lineage B, 99% in ML, 100% in MP).

Lineage A consisted of all haplotypes of the NS-morph and haplotype A09 of the ON-morph collected from NCM. There were two predominant haplotypes in lineage A, A01 and A06. Haplotypes A01 and A06 were common to NLK and GLK, and GLK and SHN populations, respectively, whereas the minor haplotypes were not shared by different populations. Haplotype A09, detected in the ON-morph from NCM, was highly diverged from A01–A08, being connected to the A06 haplotype with 18 substitutions.

Lineage B contained all haplotypes of the MC- and ON-morphs except for A09, and the haplotype of *B. variabilis* collected from Hong Kong. Haplotypes in this lineage were further separated into two groups, one consisting of haplotypes of the ON-morph from OLO (B08, B09 and B10), and the other consisting of haplotypes of the MC-morph (B01, B02, B03, B05, B06 and B07) and haplotype B04 of the ON-morph from NCM (Fig. 11). There were two major haplotypes in lineage B, namely, B01 of the MC-morphs and B08 of the ON-morphs; these were diverged by three substitutions. Haplotype B01 was detected in all MC-morph populations. Derivative singleton haplotypes of B01 were only detected in single marine lake populations, with the exception of haplotype B03, which was detected in two populations. The haplotypes detected in the MC-morphs were closely related to each other.

The sequences of *B. variabilis* were nested within lineage B. The analysis also clearly showed that the ON-morph was not monophyletic. The ON-morph haplotypes detected from OLO and NCM were quite different. A minor haplotype, B10, in the OLO population was diverged from the B08 major haplotype by five substitutions. On the other hand, the haplotypes detected in the NCM population were more diverged. Of the two haplotypes found in NCM, haplotype B04 belonged to lineage B, whereas haplotype A09 was clearly derived from lineage A and was remarkably differentiated (18–23 substitutions) from the other haplotypes of lineage A. Haplotype B04 was more closely related to the haplotypes observed in MC-morphs, as opposed to those observed in OLO.

The other reference sequences were highly diverged from the haplotypes of the three morphs (Fig. 11). The sequence of *M. minimus* was clearly nested within the *Brachidontes–Hormomya* complex clade. Some nodes of the reference sequences were strongly supported statistically by posterior probabilities, although most of the basal nodes of the reference sequences were not supported.

The haplotypes of M-type genes formed a highly distinctive clade, which was the sister group to a clade consisting of three reference species, *M. minimus* (Italy, Mediterranean), *B. exustus* (Panama, Atlantic) and *B. adamsianus* (Mexico, Pacific) (Fig. 11). The clade consisting of the M-type haplotypes and the three reference species was strongly supported in Bayesian and ML analysis, but not in MP analysis.

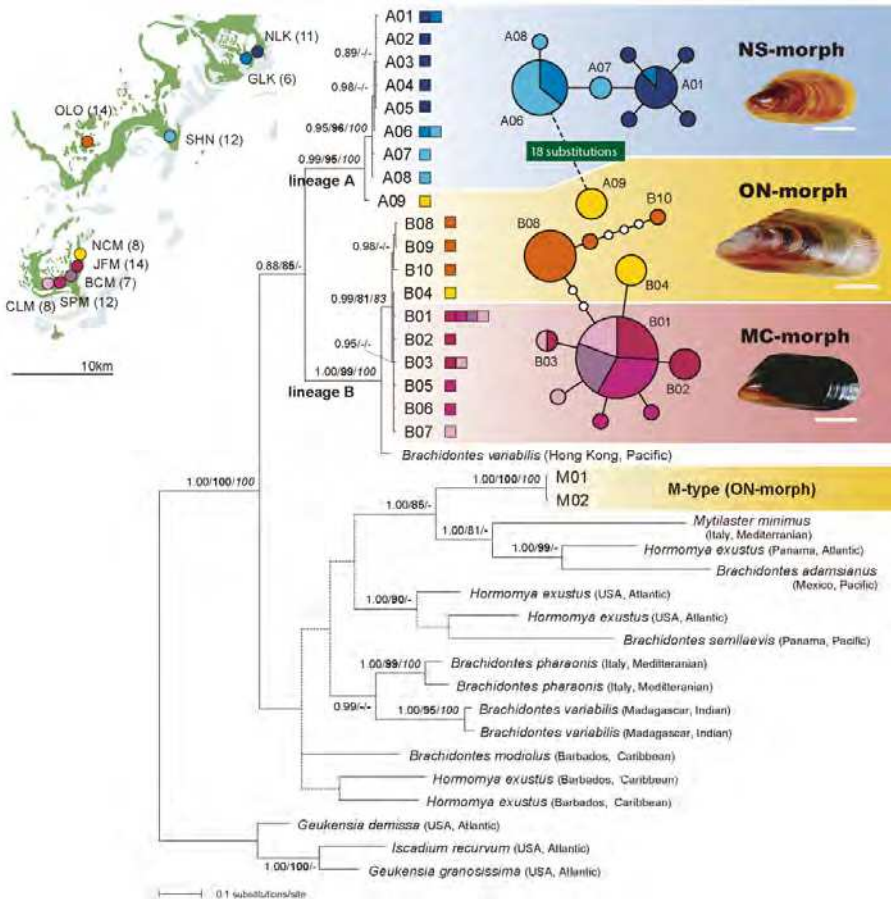


Fig. 11. Phylogenetic relationships among three morphs of marine lake mussels and *Brachidontes-Hormomya* complex. The Bayesian tree using the best-fit model for the COI dataset (GTR I+G), partitioned into the three codon positions is shown. Bayesian clade posterior probabilities (> 0.80 , regular) are indicated with ML (> 80 , bold) and MP (> 80 , italic) bootstrap values at each branch. Squares beside the haplotype symbols indicate the sampling site where the haplotype was found. Topologies of the three trees did not agree with each other in parts within clades consisting of closely related haplotypes. Statistical parsimony network was constructed based on the F-type COI gene haplotypes. Circle size corresponds to the comparative frequency of the haplotypes. Small white circles show missing haplotypes that were not detected. The sequences of *Brachidontes exustus* were registered as *Hormomya exustus* in the DNA databases and the registered name was used here (From T. V. Goto et al., *Zool. Sci.* 28:568-579, 2011).

Genetic diversity of marine lake mussels Haplotype diversity (h) and nucleotide diversity (π) in each marine lake population are shown in Table 7. Haplotype diversity in each population ranged from 0.000 in the BCM population to 0.618 in the NLK population. Nucleotide diversity in all populations, except for NCM, was quite low (0.000–0.001). The nucleotide diversity was highest (0.086) in the NCM population because of its highly diverged haplotypes.

We further calculated pairwise Φ_{ST} as an index of genetic differentiation between the marine lake populations (Table 8). The Φ_{ST} values were statistically significant ($p < 0.05$), except for between the four populations on Mecherchar Island (JFM, BCM, SPM and CLM). The other exception was for GLK and SHN, which are located on separate islands, approximately 10 km apart. The values between populations of different morphs were higher than those between populations of the same morph. Values for both Tajima's D and Fu's F_s were significantly negative only in the NLK population at the 95% level ($p = 0.020$ and 0.001 , respectively), suggesting a recent rapid demographic expansion. Similarly, in the SPM and CLM populations, negative values of Fu's F_s ($p = 0.025$ and 0.043 , respectively) suggest rapid expansion, but Tajima's D was not significant at the 95% level ($p = 0.063$ and 0.085 , respectively).

Morph	NS-morph			ON-morph				MC-morph				
	NLK	GLK	SHN	Whole	OLO	NCM	Whole	JFM	BCM	SPM	CLM	Whole
Sampling sites	38	15	5		4	4		30	22	14	30	
Depth of marine lake (m)	11	6	12	29	14	8	22	14	7	12	8	41
Sample size for genetic analysis	5	2	3	8	3	2	5	3	1	3	3	6
Number of F-type haplotypes	0.618	0.333	0.439	0.704	0.275	0.571	0.662	0.539	0.000	0.318	0.464	0.387
Haplotype diversity (h)	1.310	1.201	0.846	2.459	1.247	86.486	48.633	1.049	0.000	0.601	0.901	0.760
Nucleotide diversity (π) (10^{-3})	4/0	2/0	2/0	7/0	4/0	60/24	63/24	2/0	0/0	2/0	2/0	5/0
Number of Ti/Tv	-1.712	-1.132	-0.850	-0.698	-1.481	2.615	0.633	-0.201	0.000	-1.451	-1.310	-1.617
Tajima's D	0.020	0.142	0.222	0.297	0.048	1.000	0.804	0.418	1.000	0.063	0.085	0.024
p -value	-2.908	0.952	-0.725	-2.751	0.117	19.128	19.090	-0.207	0.000	-1.325	-0.999	-4.123
Fu's F_s	0.001	0.592	0.097	0.053	0.424	1.000	1.000	0.308	N.A.	0.025	0.043	0.001
p -value	N. A. Not applicable.											

N. A. Not applicable.

Table 7. Number of specimens and estimated genetic diversities between populations of the three morphs of marine lake mussels based on COI genes. h : haplotype diversity, π : nucleotide diversity, number of transitions (Ti) and transversions (Tv), values of Tajima's D and Fu's F_s (Modified from T. V. Goto et al., *Zool. Sci.* 28:568-579, 2011).

Genetic divergence among three morphs of mussels F-type COI gene-based phylogenetic analysis clearly showed high genetic divergence between the NS- and MC-morphs of the marine lake mussels (Fig. 11). The genetic distances between NS- and MC-morphs (e.g. p -distance = 0.146 between A01 and B01) were comparable to those reported between species (Terranova et al., 2007) or within cryptic species (Lee and Foighil, 2004, 2005). Lee and Foighil (2004, 2005) estimated the evolutionary rates of the third-codon position for the F-type COI gene in *B. exustus* as 18.3–24.4% per million years per lineage. Applying this mutation rate to the genetic distances between the NS- and MC-morphs (p -distance = 0.378 at the third codon position between A01 and B01), we estimated the time of the NS/MC split to be approximately 0.78–1.03 million years ago. This divergence time is much older than the date of formation of the marine lakes after the Last Glacial Maximum.

Despite the remarkable morphological differences between ON- and MC-morphs, we found lower levels of genetic differentiation between these two morphs. The genetic distance between ON- and MC-morphs (average p-distance at the third codon position = 0.0053; 0.022–0.029 million years ago) was as small as the level within a species or among closely related taxa in the *M. edulis* species complex (Gérard et al., 2008). This may indicate that a rapid change can occur in shell morphology of mussels in the marine lakes. Indeed, it is known that shell characters, such as size, thickness and growth rates, of bivalves readily change according to environmental conditions (Seed and Richardson, 1990; Beadman et al., 2003; Schöne et al., 2003). The limnological conditions of OLO and NCM, which the ON-morph mussels inhabit, are different from the other seven lakes. For example, there is no anaerobic bottom water in OLO and NCM. Such differences in the local environment might have shaped the unique morphology of the mussels via some adaptation in the lakes over a relatively short period.

	NLK	GLK	SHN	NCM	OLO	JFM	BCM	SPM	CLM
NLK		0.009	0.000	0.000	0.000	0.000	0.000	0.000	0.000
GLK	0.653*		0.739	0.027	0.000	0.000	0.000	0.000	0.000
SHN	0.740*	-0.063		0.000	0.000	0.000	0.000	0.000	0.000
NCM	0.542*	0.433*	0.558*		0.000	0.000	0.027	0.000	0.036
OLO	0.991*	0.992*	0.993*	0.538*		0.000	0.000	0.000	0.000
JFM	0.992*	0.993*	0.994*	0.529*	0.822*		0.234	0.090	0.171
BCM	0.994*	0.996*	0.996*	0.404*	0.857*	0.010		0.991	0.991
SPM	0.994*	0.995*	0.995*	0.501*	0.844*	0.121	-0.051		0.622
CLM	0.992*	0.993*	0.994*	0.423*	0.821*	0.076	-0.018	0.009	

Below: Φ_{ST} . The asterisks indicate significant values ($P < 0.05$).

Above: P values.

Table 8. Estimated pairwise Φ_{ST} between marine lake populations based on COI genes (From T. V. Goto et al., *Zool. Sci.* 28:568-579, 2011).

mtDNA lineages of marine lake mussels No COI haplotype was common to all three morphs, suggesting an absence of recent gene flow between the morphs (Fig. 11). Population structures of both NS- and MC-morphs consisted of one or two major and several minor COI haplotypes, and exhibited a star-like pattern of the haplotype network, indicating a founder effect and/or bottlenecking. ON-morphs, on the other hand, showed a more complex pattern of haplotype variations: 1) no haplotype was shared between the two ON-morph populations (NCM and OLO); 2) lineage A and B coexisted in NCM, while only lineage B haplotypes were found in OLO; and 3) the lineage B haplotype in NCM (B04) was more closely related to haplotypes in populations of MC-morphs (JFM, BCM, CLM and SPM) than to the other haplotypes of ON-morphs. These features suggest that the ON-morph may have evolved through a more complicated series of historical events than the NS- and MC-morphs.

In general, the coexistence of multiple lineages of mtDNA haplotypes in a single population can be explained by either ancestral polymorphism or introgression. If the effective population size has been large and constant (or expanding) and the intervals between population splits short, ancestral polymorphisms are likely to be retained in the single population (Pamilo and Nei, 1988; Takahata, 1989). However, this may not be the case in the marine lake mussels because a small effective population size and/or past bottlenecking

were suggested by the low level of nucleotide diversity. It is rather likely that mtDNA introgression took place in NCM—the haplotypes of lineage A were possibly introduced into an ancestral population that originated from lineage B. There is another possible scenario for the multiple lineages of mtDNA in ON-morph. The OLO and NCM populations of ON-morphs do not form a monophyletic group in the mtDNA phylogeny, and are separated geographically on different islands, yet the shell morphology of mussels from the two populations is indistinguishable. As mentioned above, the limnological conditions of OLO and NCM might have shaped the unique morphology of ON-morphs through convergence. Alternatively, it is also possible that ON-morph has been formed through hybridization between the morphs, although further genetic analysis using nuclear DNA markers will be necessary to clarify this issue.

In this study, we were unable to obtain M-type sequences from NS- and MC-morphs, and the NCM population of ON-morphs. This could be attributable to either technical difficulties, or the lack of an M-type genome, such as in some *Mytilus* males (Hoeh et al., 1997; Quesada et al., 1999, 2003). The previous studies suggested that some M-Type genes were newly appeared male-transmitted mtDNA: hypothetical switch of transmitting route from an M-type to F-type. Hence, the discovery of M-type from NS- and MC-types may suggest that such evolutionary event occurred in mitochondrial genomes of the morphs.

Evolutionary aspects of marine lake mussels Genetic diversity of the marine lake mussels, inferred from the haplotype diversity ($h = 0.000-0.662$) and nucleotide diversity ($\pi = 0.000-0.002$), tended to be lower than that reported for other mussels inhabiting seashores, such as Floridian *Brachidontes* ($h = 0.546-0.987$) (Lee and Foighil, 2004, 2005), Mediterranean *Brachidontes* ($h = 0.733-1.000$; $\pi = 0.005-0.042$) (Terranova et al., 2006, 2007) and *Mytilus* collected from the northern and southern hemispheres ($h = 0.600-0.950$; $\pi = 0.002-0.021$) (Gérard et al., 2008). This indicates a small effective population size and/or past bottlenecks of the marine lake populations. Because the inhabitable area of the marine lake mussels is limited, the effective population size would likely be relatively small, potentially leading to bottlenecks.

Since a single major haplotype was common to all populations of MC-morphs, these populations appear to have been founded and diverged recently. The time of divergence corresponds to the presumptive formation time of the marine lakes, after the Last Glacial Maximum, approximately 18,000 years ago (Hamner and Hamner, 1998; Dawson and Hamner, 2005). In contrast, no haplotype was common to NLK and SHN populations of NS-morphs, and the Φ_{ST} values were statistically significant ($p < 0.05$) between them. Therefore, founder populations of NLK and SHN may have slightly differentiated from each other before colonization of the lakes. This prior genetic differentiation may have been the result of geographic separation of the ancestors. It should be noted, however, that the major haplotype found in GLK was also present in SHN but not NLK, despite GLK being geographically closer to NLK than SHN. Therefore, the level of genetic differentiation between populations does not necessarily correspond to geographical distance separating the marine lakes.

In conclusion, our data clearly indicate that the morphs of the marine lake mussels are differentiated in both morphology and mtDNA lineages. This is especially the case for NS- and MC-morphs, which are differentiated from each other to the level of species. In contrast,

the relatively low level of genetic divergence between ON- and MC-morphs suggests that these morphs have rapidly acquired different morphological characters. The present study provides empirical evidence of diversification of mussels in isolated marine environments. Analysis of more sensitive nuclear genetic markers and the identification of additional variant M-type haplotypes of mtDNA markers will assist in better clarifying the evolutionary history of the marine lake mussels.

3.4 Perspective on evolution in the marine lakes

As shown by the examples of a cardinal fish and mussels in the marine lakes, the marine animals in each isolated marine lake have undergone a peculiar evolutionary process, even during a short evolutionary period of 5,000–12,000 years. On the basis of the data for the genetic diversity and differentiation of the animals in the marine lakes, we can predict their future evolutionary patterns after isolation in each marine lake. Fig. 12 (a) shows that individuals of the ancestral lagoon populations are isolated in some meromictic marine lakes and are driven to extinction by severe environmental changes and pathogenic infections. As shown in Table 2, the fact that some diagnostic species do not inhabit some meromictic lakes strongly supports this case. Fig. 12 (b) shows that genetic differentiation does not occur among the marine lake populations, and this is actually observed in most of the holomictic marine lakes. Fig. 12 (c) shows the following situation. Populations isolated in meromictic marine lakes are genetically differentiated during some extent of evolutionary time; however, the meromictic lakes turn into holomictic lakes following the collapse of limestone, individuals from the marine lake populations make secondary contact with those of the lagoon populations, and genetic mixing occurs between the marine lake and lagoon populations. In this case, the genetic diversity of the mixed population must be much higher than that of the marine lake population. Fig. 12 (d) shows that populations that have been isolated in meromictic lakes for a long evolutionary time and are largely genetically differentiated, undergo changes in their characteristics and speciate. The 5 *Mastigias* subspecies inhabiting different marine lakes, described by Dawson (2005), must fit in this case. Recently, a living fossil eel belonging to a new family in the order Anguilliformes was described from an undersea cave in Palau (Johnson et al., 2011). The divergence time between the new species and other eels was surprisingly estimated at 200 million years ago from phylogenetic analysis based on mitochondrial genome sequences, indicating the possibility that this species has been isolated in the undersea cave and speciated. It is known that there are many undersea limestone caves in Palau and some of the caves are probably, geologically, very old (personal communication from Carp Corporation staff). Atolls composed of limestone have probably uplifted and depressed repeatedly over geological time, and marine lakes and undersea caves have been also formed and collapsed repeatedly. Various marine organisms may have speciated in such habitats and some of them may have undergone extinction.

Thus, the “marine lake model as an isolated marine environment” we present here is readily available to study the evolution of various marine species. We have continuously conducted evolutionary studies of other marine organisms including algae and microbes using genetic analyses in the marine lakes of Palau. Further studies on the adaptive evolution of marine organisms in the marine lakes are in progress.

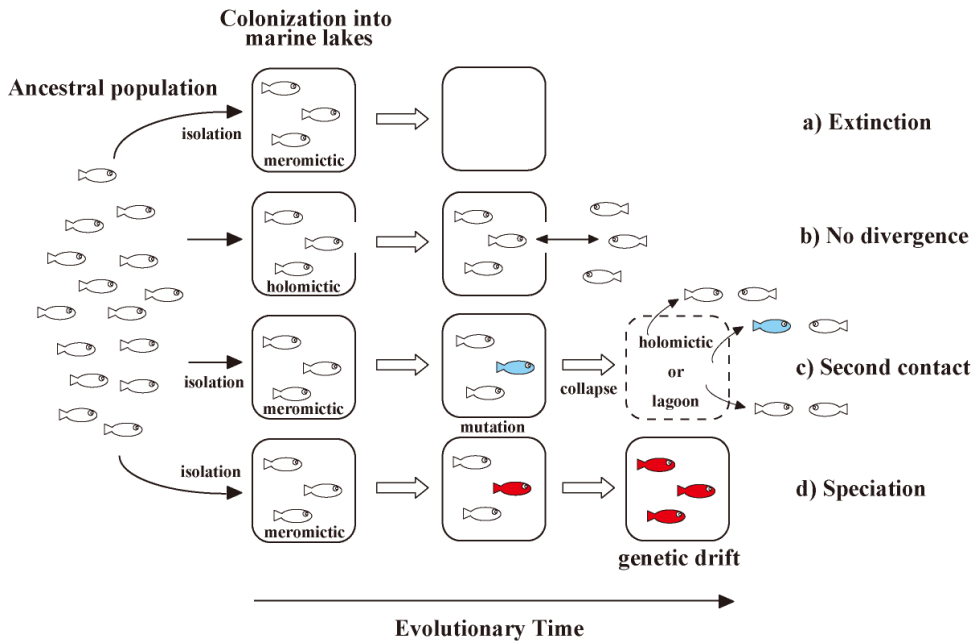


Fig. 12. Schematic figures of the evolutionary patterns in the marine lake populations

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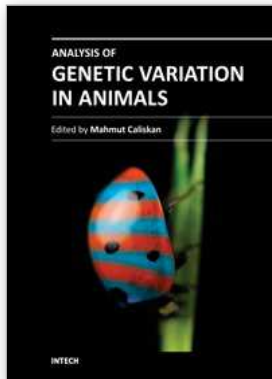
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Analysis of Genetic Variation in Animals includes chapters revealing the magnitude of genetic variation existing in animal populations. The genetic diversity between and within populations displayed by molecular markers receive extensive interest due to the usefulness of this information in breeding and conservation programs. In this concept molecular markers give valuable information. The increasing availability of PCR-based molecular markers allows the detailed analyses and evaluation of genetic diversity in animals and also, the detection of genes influencing economically important traits. The purpose of the book is to provide a glimpse into the dynamic process of genetic variation in animals by presenting the thoughts of scientists who are engaged in the generation of new idea and techniques employed for the assessment of genetic diversity, often from very different perspectives. The book should prove useful to students, researchers, and experts in the area of conservation biology, genetic diversity, and molecular biology.

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