Open Journal of Marine Science, **2013, 3, 182-189**
http://dx.doi.org/10.4236/ojms.2013.34021 Published Online October 2013 (http://www.scirp.org/journal/ojms)

Mitochondrial DNA Markers for PCR-Based Phylogenetic Analysis of Ark Shells

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Received July 29, 2013; revised August 31, 2013; accepted September 12, 2013

ABSTRACT

Arcidae species are commercially important bivalves in Japan and are commonly referred to as bloody ark due to their red blood. They have thick shells with distinct radiating ribs, and the numbers of these ribs are important morphological features for species discrimination. However, some Arcidae species are morphologically indistinguishable, with a similar number of the ribs in adults and deficient rib formation, particularly among juveniles. Thus, we developed a reliable molecular marker to genetically discriminate between 7 Arcidae species belonging to *Scapharca*, *Anadara*, and *Tegillarca* based on species-specific polymorphic segments of mitochondrial DNA. PCR amplification of partial COI, 16S rRNA, 12S rRNA, and Cyt *b* genes was performed on 7 species using 8 primer sets. Only the set of *Scapharca*-specific forward primer and universal reverse primer for the partial COI gene successfully yielded single PCR products from all 7 species examined. Thus, nucleotide sequences of 481 bp portion of these PCR products were determined, and the degrees of nucleotide substitutions ranged from 0.4% between *S. broughtonii* and *T. granosa* to 20.2% between *S. satowi* and *A. antiquata*. In addition, a phylogenetic tree showed significant differences between 7 species, with higher bootstrap support than 69.

Keywords: Ark Shell; Arcidae; Mitochondrial DNA; COI Gene; Phylogenetic Analysis

1. Introduction

Ark shells are a group of bivalves, in which red blood cells circulate through open blood-vascular systems, and hemoglobin pigment in both blood and tissue cells colors the body red [1]. Approximately 200 species of ark shells have been classified into the family Arcidae [2] and widely distributed globally in shallow tropical and temperate seas [3]. Arcidae is characterized by several derived characters including the duplivincular ligament, taxodont dentition, and shell microstructures consisting of outer crossed lamellar and inner complex crossed lamellar layers [4]. Many Arcidae species have considerable commercial value. In particular, *Scapharca*, *Anadara*, and *Tegillarca* have been actively collected for food throughout Asia [5]. In Japan, *Scapharca broughtonii*, *S. satowi*, *S. kagoshimensis*, *S. inaequivalvis*, *S. globosa ursus*, *Anadara antiquate*, and *Tegillarca granosa* are consumed by humans (**Figure 1**). Ark shells vary in size and are mostly elongate or squarish in shape. Shell surfaces are sculptured by radial ribs and covered with thick velvety periostracum [6]. These morphological features are important indicator of species identification, although the radial ribs are often similar between species (**Table 1**). Hence, morphologically identification of sympatrically related species is difficult, because their radial ribs are similar in number and change with maturity. Hence, genetic markers are required for accurate ark shells identification.

The use of molecular techniques to analyze relationships between species and populations has become widespread, and nucleotide sequence variations in the mitochondrial DNA (mtDNA) offer a powerful tool in molecular phylogenetics of marine organisms [7]. MtDNA markers have been widely used for discrimination of closely related species [8] and also for analyses of population genetic structure [9], however, there are few reports on the genetic studies of Arcidae. Such genetic data are of particular interest because Arcidae may reveal molecular evidence of gene flow or genetic isolation, which is undetectable using traditional morphological studies. In this study, genetic differences in the mtDNA cytochrome *c*

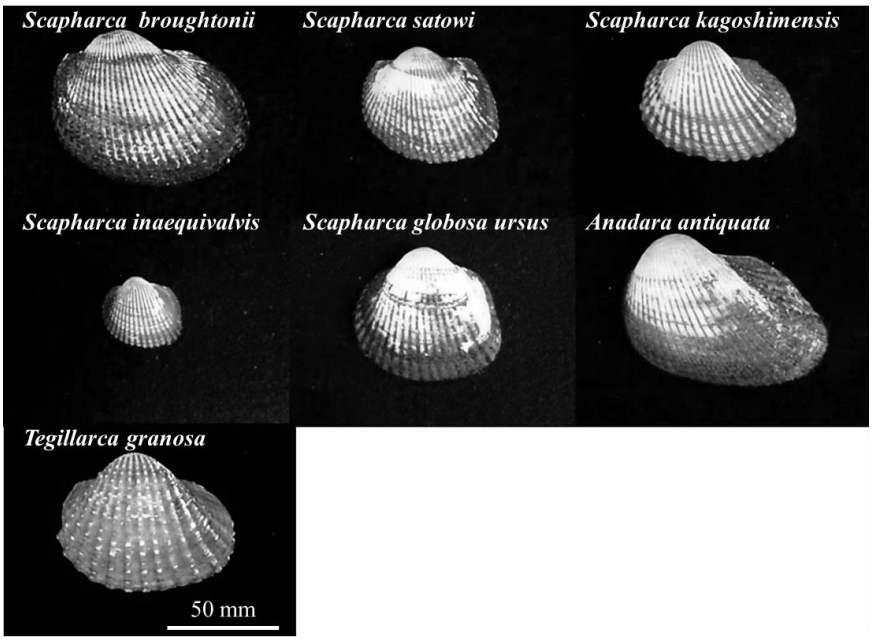

Figure 1. Photos of left valves of 7 Arcidae species.

Table 1. Morphological and geographical characterization of 7 Arcidae species based on Okutani [6] and sample profiles in this study.

Species	Approximate number of radial ribs	Size of adult individual	Distribution in Japan	Collection Site	Shell		
					SL (mm)	SH (mm)	SW (mm)
Scapharca broughtonii	42	Large	Southern Hokkaido to Kyushu	Kagawa	72.94	56.05	41.80
Scapharca satowi	38	Medium	Boso Peninsula to Kyushu	Shimane	66.71	47.23	44.73
Scapharca kagoshimensis	32	Medium	Tokyo Bay to Kyushu	Saga	35.71	28.01	29.20
Scapharca inaequivalvis	32	Medium	Boso Peninsula to Kyushu	Oita	11.48	10.29	6.55
Scapharca globosa ursus	34	Medium	Ariake Sea and Omura Bay	Kumamoto	28.78	24.92	18.55
Anadara antiquata	38	Medium	Okinawa	Okinawa	62.13	46.12	41.72
Tegillarca granosa	20	Medium	Ise Bay to Kyushu	Saga	44.60	34.91	30.39

oxidase subunit I (COI), 16S ribosomal RNA (16S rRNA), cytochrome *b* (Cyt *b*), and 12S ribosomal RNA (12S rRNA) genes between *Scapharca*, *Anadara*, and *Tegillarca* species were investigated using PCR amplification and subsequent sequence analysis to develop markers that discriminate between Arcidae species in Japan.

2. Materials and Methods

2.1. Samples

A total of 32 specimens were collected from May 2008 to November 2009, and their shell characteristics were recorded (**Table 1**). Adductor muscle was obtained from each specimen and immediately stored at −20˚C until DNA extraction.

2.2. DNA Extraction

High quality total genomic DNA was prepared from small scraps of frozen adductor muscle according to the modified urea-SDS-proteinase K method [10-12]. 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 μg proteinase K at 55˚C and 5 M NaCl was then added and mixed. DNA was isolated with phenol-chloroform-isoamyl alcohol and subsequent 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 regions was performed in GoTaq Green PCR Master Mix (Promega) containing 2 mM $MgCl_2$, 0.5 μM each primer listed in **Table 2**, and template DNA in a Techgene thermal cycler (Techne). PCR protocol consisted of an initial denaturation at 95˚C

Table 2. List of primer sequences.

Primer set		Primer sequence (5' to 3')	Annealing temperature (˚C)	Reference
COI-1	LCO1490 HCO2198	GGT CAA CAA ATC ATA AAG ATA TTG G TAA ACT TCA GGG TGA CCA AAA AAT CA	52	Folmer *et al.* [13]
COI-2		ATY GGN GGN TTY GGN AAY TG ATN GCR AAY TTY GGN TC	48	Matsumoto and Hayami [14]
COI-3	CGCIF CGCIR	ATT GGG GGG TTT GGT AAC TG ATT GTA AAC AAA GCA CCC AT	56	Iidzuka Unpublished
COI-4	L HCO2198	GGT GTG TGT TTA AGA TTT CAC A TAA ACT TCA GGG TGA CCA AAA AAT CA	50	Lee and Kim [2] Folmer *et al.* [13]
16S-1	16Sar 16Sbr	CGC CTG TTT AAC AAA AAC AT CCG GTC TGA ACT CAG ATC ATG T	50	Pulmbi *et al*. [15]
16S-2	16Sar 16Sbr	CGC CTG TTT ATC AAA AAC AT CCG GTC TGA ACT CAG ATC ACG T	54	Kessing *et al.* [16]
Cytb-1	UCYTB151F UCYTB272R	TGT GGR GCN ACY GTW ATY ACT AA GCR AAN AGR AAR TAC CAY TC	48	Merritt *et al*. [17]
12S-1	SSU-1 SSU-2	GTG GAT CCA TTA GAT ACC C ACT GGT ACC TTG TTA CGA CTT	45	Berschick [18]

for 2 min, followed by 40 cycles of 10 sec at 95˚C, 20 sec at annealing temperature listed in **Table 2**, and 40 sec at 72˚C, and a final extension at 72˚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. Phylogenetic Analysis

Nucleotide sequencing of double strands of PCR product was accomplished using a BigDye Terminator version 3.1 Cycle Sequencing Kit (Applied Biosystems) with an automated 3730xl DNA Analyzer (Applied Biosystems). Nucleotide sequences of PCR products were aligned using CLUSTAL W [19] in MEGA version 5.5 [20] and deposited in GenBank (*Scapharca broughtonii*, AB690346; *S. satowi*, AB690347; *S. kagoshimensis*, AB690348; *S. inaequivalvis*, AB690349; *S. globosa ursus*, AB690350; *Anadara antiquata*, AB690351; and *Tegillarca granosa*, AB690352). A neighbor-joining tree with bootstrap analysis was constructed from 10,000 replicates based on the Kimura 2-parameter distance [21] using *Cucullaea labiata* (AB050892) and *Barbatia fusca* (AB050899) as outgroups.

3. Results

PCR tests were performed using 8 primer pairs to verify useful regions of the mtDNA for the phylogenetic analysis of 7 Arcidae species (**Table 3**).

Firstly, a universal primer pair COI-1 developed for PCR amplification of the mtDNA COI gene was examined. This primer pair amplified the mtDNA COI gene from 4 Arcidae species such as *S. broughtonii*, *S. globosa ursus*, *A. antiquata*, and *T. granosa*. Secondly, primer pairs COI-2 and COI-3 developed for PCR amplification of the bivalve the mtDNA COI gene were examined. Neither of these primer pairs amplified the mtDNA COI gene from 7 Arcidae species. Thirdly, a unique primer pair COI-4 developed for PCR amplification of *Scapharca* mtDNA COI gene was examined. The forward primer of COI-4 was designed as a nested PCR primer inside of the forward primer of the COI-1 [2]. This primer pair successfully amplified the mtDNA COI gene from 7 Arcidae species (**Figure 2**). Finally, universal primer pairs developed for PCR amplification of the mtDNA 16S rRNA, 12S rRNA, and Cyt *b* genes were examined. The primer pairs of 16S-1, 16S-2, and Cyt-1 did not consistently amplify the respective genes from 7 Arcidae species, though a small amount of the PCR product was obtained from *S. kagoshimensis* by Cyt-1. A fragment of approximately 630 bp was amplified from the mtDNA COI gene in all 7 Arcidae species using the unique primer pair COI-4, and a 481 bp portion of complementary nucleotide sequence was obtained after correction and alignment. The average base composition of these sequences was calculated to be A—25.10%, T—37.84%, C—15.95%, and G—21.12%. Consistent with the known composition of mtDNA, this base composition was biased towards adenine and thymine (62.94%). Alignment of the nucleotide sequences of the mtDNA COI gene of 7 Arcidae species revealed a relatively high level of variability, and 111 variable sites were determined from a total of 481 sites. Of 111 variable sites, there were 73 transitions and 45 transversions, and neither insertion nor deletion. In addition, 39 variable sites at the first or second codon positions occurred 31 nonsynonymous amino acid substitutions (**Table 4**). The lowest and highest degrees of nucleotide sequence substitution were calculated to be 0.4% between *S. broughtonii* and *T. granosa* and 20.2% between *S. satowi* and *A. antiquata*, respectively (**Table 5**). Phylogenetic analysis using *Cucullaea labiata* and *Barbatia fusca* as outgroups

Table 3. PCR amplification of 7 Arcidae species.

Species	MtDNA							
	COI				16S rRNA		Cyt *b*	12S rRNA
	COI-1	COI-2	COI-3	COI-4	16S-1	16S-2	Cytb-1	12S-1
Scapharca broughtonii	○	×	×	○	×	×	×	
Scapharca satowi	×	×	×	○				
Scapharca kagoshimensis	×	×	×	○	×	×	Δ	×
Scapharca inaequivalvis	×	×	×	○				
Scapharca globosa ursus	○	×	×	○	×	×	×	
Anadara antiquata	○	×	×	○	×	×	×	
Tegillarca granosa	○	×	×	○	×	×	×	

Open circle, open triangle, cross mark, and blank represent amplification of all specimens, amplification of a part of specimens, no amplification, and not tested, respectively. Primers are listed in **Table 2**.

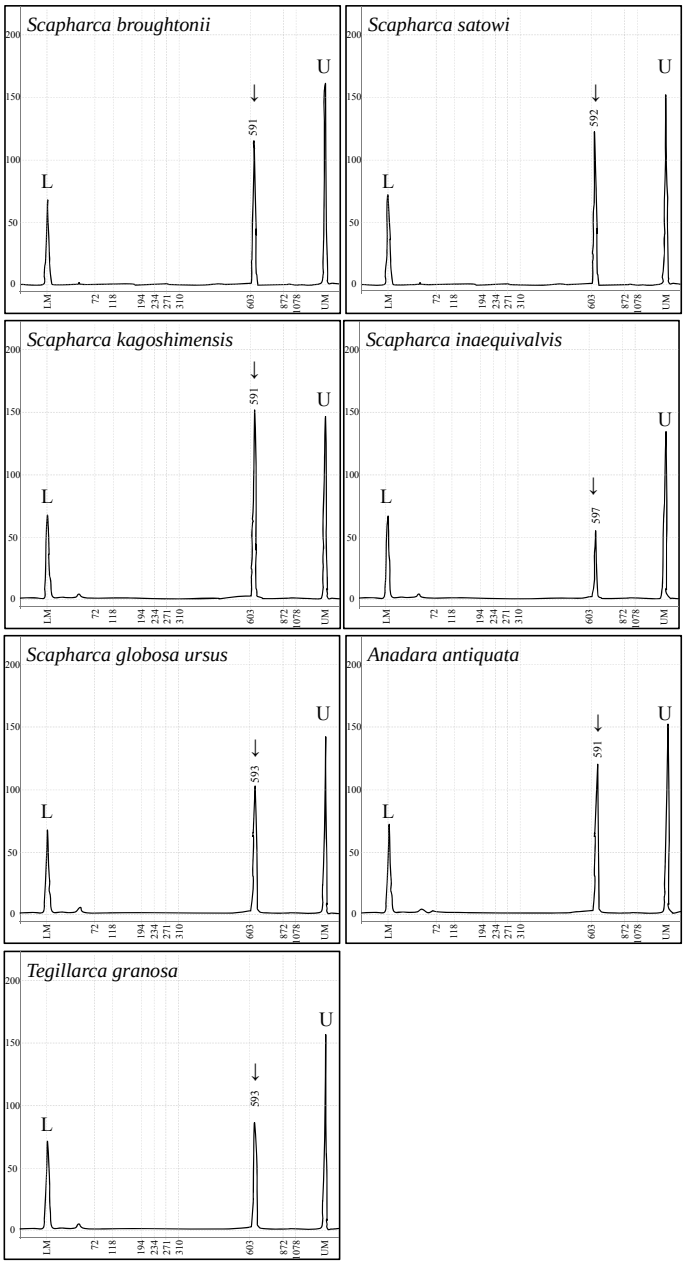

Figure 2. Electropherograms for PCR amplification of the mtDNA COI gene from 7 Arcidae species. L, lower marker; U, upper marker; arrow, PCR product.

Table 4. Variable sites of the mtDNA COI gene of 7 Arcidae species.

Species	Nucleotide variable site																												
	2	4	17	19	20	23	26	28	31	32	35	39	41	44	49	50	51	52	56	59	62	65	68	71	72	74	85	86
Scapharca broughtonii	G	T	T	G	T	T	G	T	T	G	C	C	T	T	A	A	A	G	C	A	A	T	T	T	C	A	G	T
Scapharca satowi	A	.	.	.	.	.	A	.	.	A	A	.	G	C	G	.	T	T	T	G	T	.	G	G	T	G	A	A
Scapharca kagoshimensis	A	.	C	.	A	C	A	.	.	.	.	T	.	.	G	G	.	.	.	.	T	C	.	.	T	G	.	G
Scapharca inaequivalvis	A	.	C	A	A	C	A	.	G	.	.	T	.	.	G	G	.	.	.	.	T	C	.	.	T	G	.	G
Scapharca globosa ursus	A	.	C	.	A	.	A	A	G	.	.	T	.	G	G	G	.	.	.	.	T	C	C	.	T	.	.	G
Anadara antiquata	A	A	C	.	A	C	A	.	G	.	.	T	.	G	G	G	.	.	.	.	T	C	C	.	T	G	.	G
Tegillarca granosa	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.
		NS		NS					NS			NS		NS	NS		NS										NS	
codon position	3	2	3	2	3	3	3	2	2	3	3	1	3	3	2	3	1	2	3	3	3	3	3	3	1	3	2	3

Species	Nucleotide variable site																											
	98	101	104	116	118	119	122	128	132	143	152	155	160	165	167	170	173	176	177	184	185	187	191	197	203	206	218	220
Scapharca broughtonii	A	A	T	G	T	A	A	T	T	G	A	T	G	T	A	T	A	T	T	G	G	G	C	T	T	T	G	T
Scapharca satowi	.	T	G	.	G	G	T	C	A	A	G	C	.	C	.	.	.	A	.	A	A	.	T	.	C	A	A	G
Scapharca kagoshimensis	G	.	A	A	.	.	T	.	.	A	.	.	.	.	G	.	.	C	C	.	C	A	T	.	.	.	.	G
Scapharca inaequivalvis	G	.	A	A	.	.	T	.	.	A	.	.	.	.	G	C	.	C	C	.	C	.	T	A	C	G	.	G
Scapharca globosa ursus	G	.	A	.	.	.	.	.	.	.	.	.	.	.	G	.	.	C	.	.	.	.	T	.	.	.	.	G
Anadara antiquata	G	.	A	A	A	.	T	.	.	A	.	.	A	.	G	.	C	C	C	.	C	A	T	A	.	G	.	G
Tegillarca granosa	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.
					NS		NS		NS											NS				NS				NS
codon position	3	3	3	3	2	3	3	3	1	3	3	3	2	1	3	3	3	3	1	2	3	2	3	3	3	3	3	2

Species	Nucleotide variable site																											
	221	230	236	239	242	245	246	251	256	259	262	267	275	277	278	281	284	286	294	295	298	299	307	314	317	322	325	332
Scapharca broughtonii	A	C	T	T	T	A	C	G	T	T	G	G	C	A	A	T	A	T	A	C	G	G	T	T	T	G	T	T
Scapharca satowi	G	T	.	.	A	G	.	A	.	.	.	.	T	T	.	.	.	.	.	G	A	A	C	C	.	.	.	A
Scapharca kagoshimensis	G	.	.	C	A	.	T	.	.	.	.	T	T	T	G	C	G	.	G	.	.	.	.	.	.	A	.	.
Scapharca inaequivalvis	G	.	.	C	A	.	T	.	.	.	.	T	T	T	G	C	.	.	G	.	.	.	.	.	.	.	.	.
Scapharca globosa ursus	G	.	.	C	A	.	T	.	.	.	.	T	T	T	.	C	.	.	C	.	.	.	.	.	.	.	.	.
Anadara antiquata	G	.	A	C	A	.	T	.	A	A	T	T	T	T	.	C	.	A	G	.	.	.	.	.	G	A	A	.
Tegillarca granosa	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.
			NS				NS		NS	NS	NS	NS		NS				NS	NS		NS		NS			NS	NS	
codon position	3	3	3	3	3	3	1	3	2	2	2	1	3	2	3	3	3	2	1	2	2	3	2	3	3	2	2	3

Species	Nucleotide variable site																											
	341	344	347	357	362	369	371	374	383	385	386	392	408	409	410	413	419	422	443	446	449	452	455	458	460	464	471	
Scapharca broughtonii	G	A	A	T	A	C	A	T	T	T	G	T	T	T	A	G	T	T	T	T	T	G	T	T	G	T	C	
Scapharca satowi	.	.	.	.	.	T	.	A	.	.	A	G	.	.	.	.	.	C	.	.	.	A	G	G	.	.	T	
Scapharca kagoshimensis	A	G	G	.	G	T	G	.	.	.	.	C	C	.	G	.	.	.	C	.	.	A	G	C	A	C	T	
Scapharca inaequivalvis	A	G	G	C	G	T	G	.	.	G	.	C	C	.	G	.	C	.	C	C	.	A	G	C	A	C	T	
Scapharca globosa ursus	A	.	G	.	G	T	G	.	.	G	.	C	C	.	G	.	.	.	C	C	.	.	G	G	.	C	T	
Anadara antiquata	A	.	G	.	G	T	G	.	G	G	.	C	C	G	G	A	C	C	C	C	C	A	G	C	A	C	T	
Tegillarca granosa	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
				NS					NS					NS											NS			
codon position	3	3	3	1	3	1	3	3	1	2	3	3	1	2	3	3	3	3	3	3	3	3	3	3	2	3	1	

Dots represent matches with nucleotides present in *Scapharca broughtonii*. NS and under lines are nonsynonymous substitution site and intraspecific variation, respectively. Numbers refer to positions of base pairs from the start to fragment.

Table 5. Pairwise sequence divergences of the mtDNA COI gene of 7 Arcidae species.

Species		Nucleotides						
		1	2	3	4	5	6	7
1	*Scapharca broughtonii*		0.135	0.112	0.139	0.106	0.172	0.004
2	*Scapharca satowi*	59		0.165	0.176	0.162	0.202	0.135
3	*Scapharca kagoshimensis*	49	70		0.032	0.043	0.065	0.112
4	*Scapharca inaequivalvis*	60	74	15		0.045	0.054	0.134
5	*Scapharca globosa ursus*	47	69	20	21		0.070	0.106
6	*Anadara antiquata*	73	84	30	25	32		0.166
7	*Tegillarca granosa*	2	59	49	58	47	71	

Above and below diagonal indicate mean distance values calculated by the Kimura's two-parameter distance method and number of nucleotide substitutions, respectively.

suggested that 7 Arcidae species formed a monophyletic clade, which was supported by higher bootstrap values than 69. These analyses additionally indicated that *A. antiquata* and *T. granosa* could be the sister group to *Scapharca* species (**Figure 3**).

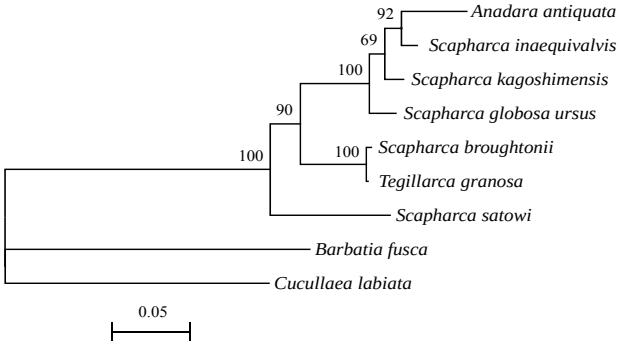

Figure 3. Neighbor-joining tree of 7 Arcidae species with *Barbatia fusca* and *Cucullaea labiata* as outgroup inferred from the mtDNA COI gene. Bootstrap values higher than 50 are shown at nodes.

4. Discussion

In recent years, bivalve resources have been threatened by overfishing or ecological changes, resulting in decreases in wild stocks in Japan [22]. In an effort to promote stock restoration, bivalve seeds were imported and released into coastal waters for farming, and seeds of many mollusk species including *S. broughtonii* and *S. subcrenata* were mixed with clam seeds and released into fishing areas of Japan [23].

Despite the commercial importance of Arcidae in Japan, to date little is known about its genetic characteristics and phylogenetic relationships in the native range [24]. Recent developments of DNA markers and genetic identification keys have helped to resolve questions surrounding bivalve taxonomic profiles. Molecular diagnostic markers were specifically developed to identify *S. broughtonii* in Korea and China [25,26], but these remain unavailable in Japan. In addition, molecular diagnostic markers for discriminating sympatrically distributed, closely related species belonging to Arcidae was also unavailable. In this study, we developed a reliable molecular diagnostic marker, which genetically discriminates between 7 Arcidae species belonging to *Scapharca*, *Anadara*, and *Tegillarca* based on the species-specific polymorphic segments of mtDNA COI gene.

Lee and Kim [2] reported genetic differentiation among *S. broughtonii*, *S. satowi*, and *S. subcrenata* in Korea based on a 599 bp portion of the mtDNA COI gene. The number and degree of nucleotide sequence variations among these 3 *Scapharca* species ranged from 27% to 48% and from 4.7% to 12.4%, respectively. Those among the same *Scapharca* species in Japan, however, ranged from 49% to 70% and from 11.2% to 16.5%, respectively, despite the shorter 481 bp portion of the corresponding the mtDNA COI gene (**Table 5**). These results suggest that genetic differentiation among *S. broughtonii*, *S. satowi*, and *S. subcrenata* may have progressed in Japan more than in Korea.

Matsumoto and Hayami [24] reported genetic relationship among *S. broughtonii*, *S. satowi*, *S. subcrenata*, *Diluvarca tricenicosta*, and *Tegillarca granosa* in Japan, *A. antiquata* in Philippine and *T. nodifera* in China based on deduced 302 amino acid sequences of the mtDNA COI gene. The neighbor-joining tree formed a monophyletic clade with *Scapharca*, *Diluvarca*, *Anadara*, and *Tegillarca* with high bootstrap support.

In the present study, **Figure 3** showed a monophyletic clade including *Scapharca*, *Anadara*, and *Tegillarca*, using a neighbor-joining tree. These nucleotide and amino acid sequences of the mtDNA COI gene indicated a monophyletic clade of closely related genera that belong to Anadarinae. In conclusion, the mtDNA COI gene was verified as a sensitive DNA marker for species identification of Arcidae species. Although DNA sequencing is undoubtedly the most powerful approach, it is costly and time-consuming. Single nucleotide polymorphisms (SNPs) genotyping has been extensively used for species identification, even in bivalves [27]. We accordingly determined several sites containing species-specific SNPs, and

developed a simple and easily accessible SNPs genotyping method to identify Arcidae species using species-specific PCR amplification of the mtDNA COI gene.

5. Acknowledgements

We thank to Dr. Ayako Yamakawa from Okinawa International University, Dr. Keiko Yamaguchi from Shimane University and Ms. Kumiko Tsuru from Oita Prefectural Agriculture, Forestry and Fisheries Research Center for providing the samples. This research was supported in part by Grant-in-Aid for JSPS Fellows Grant Number 24-2801 to Tomomi Tanaka as JSPS Research Fellow DC2, and by grants from the Agriculture, Forestry and Fisheries Council of Japan.

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