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Short Note

Genetic diversity of *Sicyopterus japonicus* as revealed by mitochondrial DNA sequencing

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Abstract — To examine the genetic diversity of *Sicyopterus japonicus*, 448 sites of the control region of the mitochondrial DNA were analyzed in 77 specimens from the four localities of Okinawa, Kochi, Wakayama, and Shizuoka in Japan. A total of 74 haplotypes were found in the individuals examined. The same haplotypes occurred in Okinawa and Kochi, Kochi and Wakayama, and two specimens in Wakayama. The average sequence in genetic characteristics within localities varied from 1.7% in Kochi, 1.6% in Wakayama to 1.4% in Okinawa. There was no significant difference in genetic characteristics among the three locations of Wakayama, Kochi, and Okinawa (10,000 times permutation test, $P>0.05$). The number of specimens from Shizuoka was too small to compare to the other three locations. The neighbor-joining tree of the mitochondrial DNA haplotypes for all specimens constructed from the Kimura's two-parameter distances suggested no evidence of genetic subdivision of *S. japonicus*. These results suggested that this species has a single panmictic population and their larvae probably have a high dispersal ability during their oceanic stage.

Key words: *Sicyopterus japonicus*, amphidromous, mtDNA, control region, population structure, larval migration, dispersal

Introduction

Amphidromous gobies are known in many taxa such as *Lentipes*, *Sicyopterus*, *Stiphodon*, *Rhinogobius* etc., and among these, the Sicydiinae is a major group with many amphidromous species (Birdsong et al. 1988). Almost all species of the Sicydiinae are widespread from the tropics to warm temperate regions. McDowall (2004) suggested that it would be Sicydiinae and other amphidromous goby groups if any tropical and subtropical freshwater fish group could be regarded as "iconic". One species that has an extremely wide geographic distribution is *Sicyopterus lagocephalus*, and this species is found longitudinally from the western Indian Ocean (Comoro Islands and Mascarene Islands) through Sri Lanka and Indonesia to the western and eastern Pacific (New Caledonia and Marquesas Islands) and latitudinally from Japan to Australia (Watson et al. 2000). Furthermore, McDowall (2003, 2004) also suggested that their marine life stages are the likely key element in explaining their distributions. There have been many biological and ecological studies on these amphidromous species of the Gobiidae in the Indo-Pacific and the Caribbean regions (see Keith 2003). However, only a few attempts have so far been made to study their marine life stages and no studies have examined the relationship between the marine life stages and the distributions of the Sicydiinae and other amphidromous gobies.

The goby, *Sicyopterus japonicus*, spawns in freshwater, and its larvae drift downstream to the sea where they spend a planktonic life before migrating back to river to grow and reproduce (Dôtu and Mito 1995). Therefore, this species of goby has an amphidromous life history (Myers 1949, McDowall 1988). *S. japonicus* has a unique behavior that enables it to climb vertical rock faces (Fukui 1979). However, only a few ecological and evolutional studies (Mochizuki and Fukui 1983, Dôtu and Mito 1995, Shen and Tzeng 2002) so far have been conducted on this species, and little attention has been specially given to its marine larval stage in this species. In the western north Pacific, *S. japonicus* is distributed from Taiwan to Fukushima prefecture (approximate species range: 2500 km) of Japan (Fig. 1) in areas along the flow route of the Kuroshio Current. The wide geographic distribution of these amphidromous gobies has resulted in speculation that their larvae may drift for thousands of kilometers, because the dispersal ability of larvae should be closely related to the species range of the adults and the population structure of these species. In order to begin to test this larval drift hypothesis, we examined the control region of the mitochondrial DNA (mtDNA) and estimated the population structure of *S. japonicus* by evaluating genetic diversity and the degree of genetic interchange among four localities throughout most of the species range of *S. japonicus*.

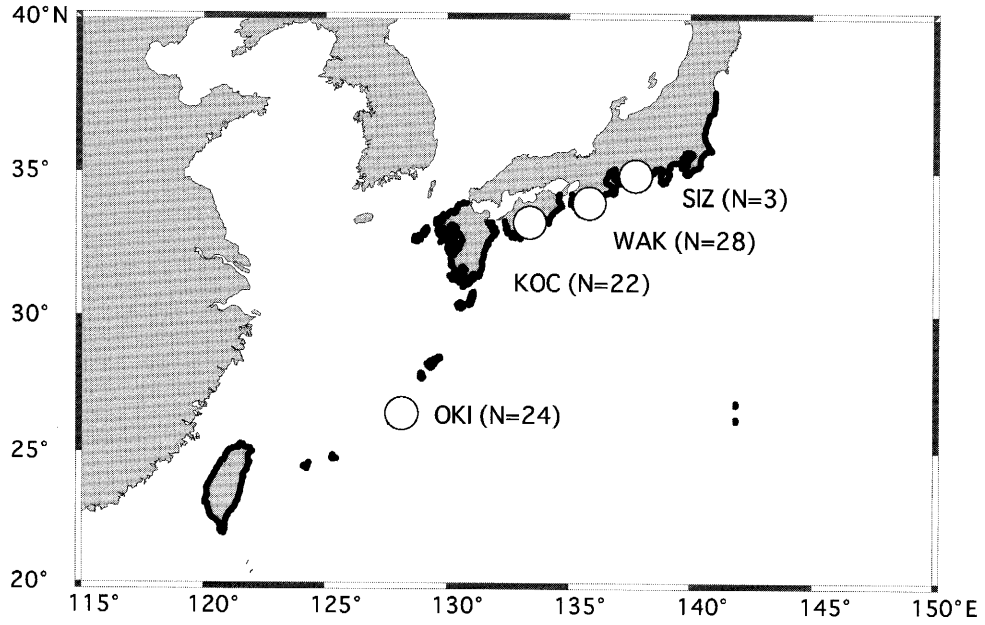

Fig. 1. Distribution of *Sicyopterus japonicus* (areas covered by thick lines) and the collection localities of specimens used in this study. The three capital letters are the abbreviations of the localities: OKI (Okinawa), KOC (Kochi), WAK (Wakayama) and SIZ (Shizuoka).

Materials and Methods

Specimens

We collected a total of 77 specimens (standard length: 44.0–126.0 mm) from Zatsun River in Okinawa (N=24), Monobe River in Kochi (N=22), Takata River, Ota River and Nagano River in Wakayama (N=28) and Nishijinden River in Shizuoka (N=3) from 27 June to 1st October 2003. The specimens were preserved in 95% ethanol after measuring their standard length. A piece of right pectoral fin was minced in a buffer consisting of 8 M urea, 10 mM tris-HCl ph 8.5, 125 mM NaCl, 50 mM EDTA and 1% volume/weight SDS (Aoyama and Tsukamoto 1997).

mtDNA amplification and sequencing method

Total genomic DNA was isolated and purified twice using phenol-chloroform-isoamyl alcohol (25 : 24 : 1) with diethyl ether and then concentrated by ethanol precipitation. A fragment of the control region of the mtDNA was amplified using polymerase chain reaction (PCR) with a pair of oligonucleotide primers, L16007 (5′-CCC AAA GCT AAG ATT CAT AA-3′) (Kocher et al. 1989, as modified by Shedlock et al. 1992) and H0600 (5′-CTG TTA ACC TTA GCG CTG-3′) (Chubb et al. 1998). The control region of the mtDNA is known to be quite variable and thus has been used for the analysis of population structure in various fishes (e.g. Iguchi et al. 1999, Tabata and Taniguchi 2000, Ishikawa et al. 2001). The PCR amplifications were carried out in a thermal cycler 9700 (Applied Biosystems, Foster City, CA, USA) under the following conditions: initial 2 min denaturation at 94°C, and 35 alternating cycles of 15 s at 94°C for denaturation, 15 s at 52°C for annealing, and 30 s at 72°C for extension. A total of 3–4 μl of each PCR product was used for 1% agarose gel electrophoresis for verifying the amplified fragment length with a standard size marker (ØX 174 *Hinc* II digest; Takara Co., Tokyo, Japan). Band characterization under ultraviolet light was made after staining with ethidium bromide. The rest of the PCR products were purified by filtration with a Microcon-100 (Amicon Inc. Bedford, MA, USA). These purified products were used as the template DNA for cycle sequencing reactions performed using Dye Terminator Cycle Sequencing FS Ready Reaction Kits (Applied Biosystems), and were run on an ABI 377 DNA Sequencer (Applied Biosystems). The forward primer L16007 and the reverse primer H0600 were used for the direct cycle sequencing reaction. Sequences were obtained from both strands for verification. The final haplotype sequences were deposited in GenBank/EMBL/DDBJ (Accession numbers: AB244792–AB244815 and AB245164–AB245213).

Sequence analysis

All sequences were edited with the multiple sequence editor DNASYS (Hitachi Software Engineering Co. Ltd, Tokyo, Japan) and preliminary alignment was achieved using CLUSTAL W (Thompson et al. 1994), the output of which was later confirmed by eye.

The haplotype and nucleotide diversity of three localities, Okinawa, Kochi and Wakayama, were estimated with the program ARLEQUIN 2.000 (Schneider et al. 2000). The population pairwise Φ_{ST} among these three localities were calculated for the mtDNA data sets and these values were

statistically tested by the 10,000 times permutation test using ARLEQUIN 2.000 (Schneider et al. 2000). The three individuals in Shizuoka could not be analyzed for the haplotype diversity, the nucleotide diversity or the population pairwise Φ_{ST}.

The phylogenetic relationships among all observed haplotypes were inferred using PAUP* 4.0b10 (Swofford, 2002). The neighbor joining method based on Kimura's (1980) two-parameter distance was applied for unrooted phylogeny estimation. The reliability of the resultant topology was assessed by bootstrapping with 1000 replications.

Results and Discussion

The sequence of 448 sites of the control region was determined for 77 specimens of *S. japonicus*. There were a total of 68 variable positions (15.2% of 448 bp). According to these variable sites, a total of 74 haplotypes were found in the 77 individuals (Fig. 2). One specimen each in the Kochi and Okinawa and in the Kochi and Wakayama samples, and two specimens in the Wakayama sample showed the same sequence arrangements. The values of the overall haplotype diversity and nucleotide diversity including Shizuoka, and the

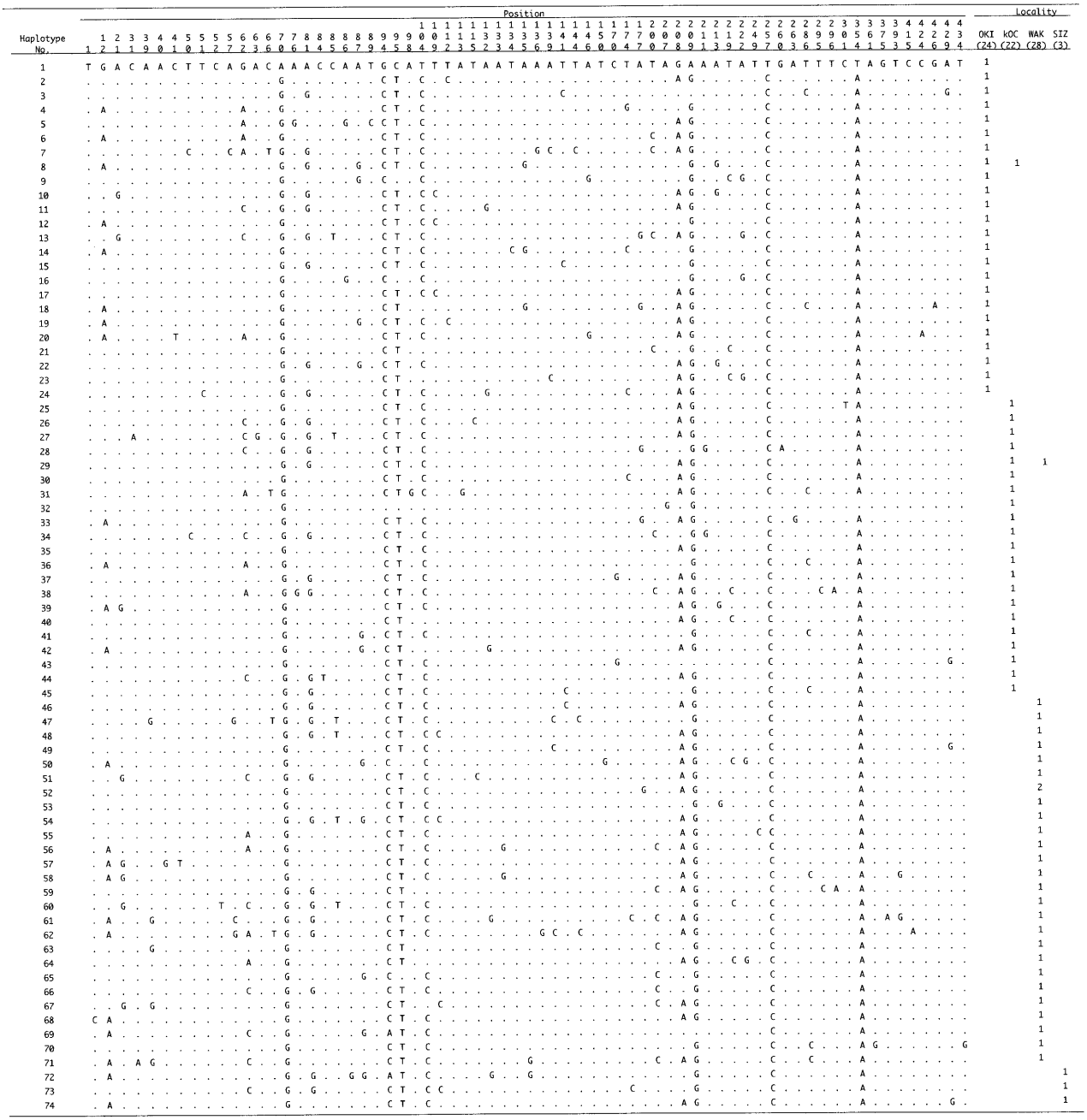

Fig. 2. Nucleotide variation and frequencies of the control region haplotypes. The dots show identical bases. Sample sizes are given in parentheses. The three capital letters are the abbreviations of the localities: OKI, Okinawa; KOC, Kochi; WAK, Wakayama; SIZ, Shizuoka.

Table 1. Haplotype diversity and nucleotide diversity overall, and at Wakayama (WAK), Kochi (KOC) and Okinawa (OKI).

Locality	Haplotype diversity	Nucleotide diversity
Overall	1.000±0.002	0.0156±0.0082
WAK	1.000±0.010	0.0162±0.0087
KOC	1.000±0.014	0.0139±0.0077
OKI	1.000±0.012	0.0169±0.0091

Table 2. Population pairwaise Φ_{ST} (above) and Φ_{ST} P values (below) estimates between *Sicyopterus japonicus* from three localities inferred from mtDNA analysis.

	Okinawa	Kochi
Kochi	−0.0136	
	0.9019±0.0029	
Wakayama	−0.0109	−0.0056
	0.8738±0.0033	0.6854±0.0047

values of each of the three localities were all the same or were very similar (Table 1). The average sequence differences within localities were 1.6% in Wakayama, 1.4% in Kochi and 1.7% in Okinawa. No significant difference for the population pairwise Φ_{ST} was detected among these three localities (10,000 times permutation test, $P>0.05$) (Table 2).

The neighbor-joining tree of the mtDNA haplotypes from all specimens examined was constructed using Kimura's (1980) two-parameter distances (Fig. 3). No long internal branches could be found, and there were no branches that supported any associations with the geographic locations.

The frequency of variable sites in the control region of mtDNA of *S. japonicus* (15.2%) is somewhat lower than those in other fishes such as the Japanese flounder, *Paralichthys olivaceus* (36%, Fujii and Nishida 1997), swordfish, *Xiphias gladius* (29%, Bremer et al. 1996), ayu, *Plecoglossus altivelis* (25%, Iguchi et al. 1999) and red sea bream, *Pagrus major* (24%, Tabata and Taniguchi 2000) and is about the same as the Japanese eel, *Anguilla japonica* (11%, Sang et al. 1994, 17%, Ishikawa et al. 2001). About 96% of the 77 individuals analyzed in this study had different haplotypes. This value is as high as Japanese flounder (98%, N=55, Fujii and Nishida 1997), Japanese eel (96%, N=82, Ishikawa et al. 2001) and ayu (90%, N=105, Iguchi et al. 1997), but is higher than swordfish (62%, N=112, Bremer et al. 1996). Similarly, the average sequence differences in the control region of *S. japonicus* (1.4–1.7%) were similar to those of the Japanese eel (1.1–1.6%, Ishikawa et al. 2001), but considerably lower than those of swordfish (3.8%, Bremer et al. 1996), Japanese flounder (4.3%, Fujii and Nishida 1997), ayu (2.2–3.2%, Iguchi et al. 1997, 1999) and red sea bream (2.7–2.8%, Tabata and Taniguchi 2000). These comparisons indicate that *S. japonicus* has various haplotypes, as in many other fishes, but the frequency of variable sites and degree of sequence difference among haplotypes are relatively lower than in other fishes except for the Japanese eel. This variability pattern may reflect the history of the *S. japonicus* population that may have included bottlenecks or rapid dispersal, so further investigation of the genetic history of *S. japonicus* population using more specimens is needed.

If there was any spatial genetic heterogeneity in *S. japonicus*, the level of genetic variability among localities would be expected to be higher than those within localities. However, the range of genetic differences within localities of this species covered those among localities, and no significant difference was found between them.

Because of this, the present study provides no support for the existence of spatial genetic heterogeneity in *S. japonicus*. The fact that the same mtDNA haplotypes were observed in different localities despite the high percentage (96.1%) of different haplotypes, also appears to be inconsistent with the multiple population model. The same haplotype being found in far distant localities, such as a pair of individuals from Kochi and Okinawa, may be evidence for a wide dispersion of larvae, even though they are not always direct offspring of one female. Furthermore, two individuals at different localities had similar mtDNA haplotypes that were greatly different from the other 75 individuals (Fig. 2 and Fig. 3). They were found at Okinawa (haplotype No. 1 in Fig. 2) and at Kochi (haplotype No. 32 in Fig. 2). Therefore, the present mtDNA analysis gives no evidence for genetic subdivision of *S. japonicus*, suggesting a single panmictic population for this species.

A lack of apparent genetic structure also has been found in some species of amphidromous gobies, but there has been evidence of genetic differentiation in some other species as well. There was no genetic structuring found among discrete island populations of five species of amphidromous gobies (*Awaous guamensis*, *Stenogobius hawaiiensis*, *Lentipes concolor*, *S. stimpsoni* and *Eleotris sandwicensis*, approximate species ranges: 600 km) in the Hawaiian Islands (Zink et al. 1996, Chubb et al. 1998). Similarly, comparative phylogeographic studies of the sympatric sister species, *Clevelandia ios* (approximate species range: 3200 km) and *Eucyclogobius newberryi*, across the California transition zone indicated that there was no genetic geographic differentiation in *C. ios*, but there was in *E. newberryi* (Dawson et al. 2001, 2002).

Genetic studies of Pandaka gobies and *Eutaeniichthys gilli* distributed on both the main Japanese Islands and the Ryukyu Islands to the south also found that there was genetic differentiation between the main islands and the Ryukyu Islands (Mukai et al. 2003, 2004). However, there was no genetic differentiation between these two areas in *S. japonicus*.

A possible factor that could help explain these various

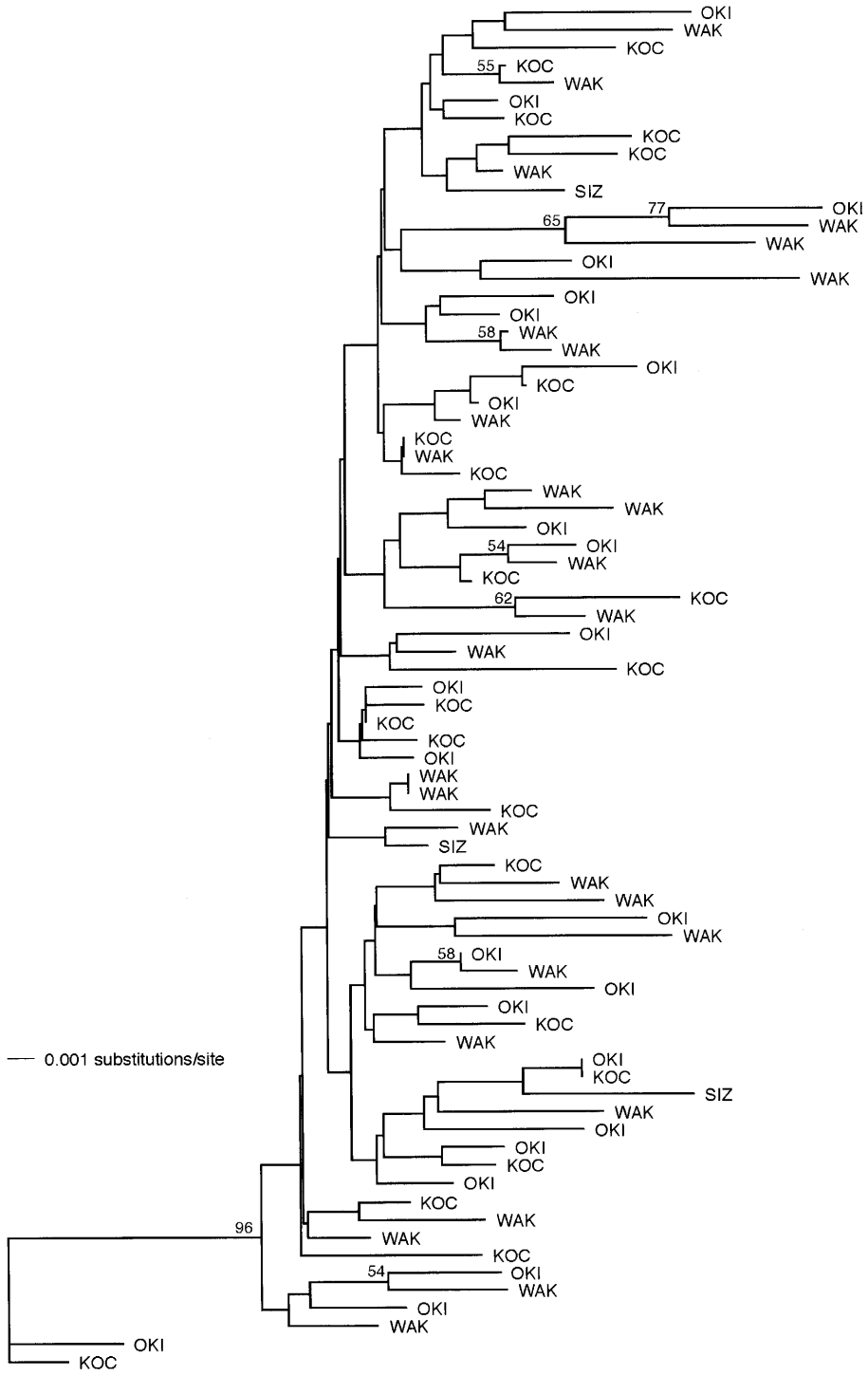

Fig. 3. Unrooted neighbor joining tree for 448 bp of the control region of mtDNA segment based on Kimura's two-parameter distances of 77 individuals. Numbers above branches are bootstrap values for 1000 replications (>50%). The three capital letters are the abbreviations of the localities: OKI, Okinawa; KOC, Kochi; WAK, Wakayama; SIZ, Shizuoka.

patterns of population structure is the duration of the marine larval phase of these species. Unfortunately, no studies have ever tried to determine the age of *S. japonicus* at the time of their movement from the sea into freshwater using daily growth rings in otoliths. Dôtu and Mito (1995) suggested that the spawning season of *S. japonicus* is from early July to early September and that their larvae ascend rivers in the next spring (March to May). This is a period of about 6 to 10 months (180 to 300 days) if the larval duration in the sea is estimated from this spawning and recruitment information. Studies of the age at the time of movement from the sea into freshwater of several Hawaiian gobies using daily growth rings in their otoliths reported ages at migration that were 150 to 169 days for *A. guamensis*, 119 to 151 days for *S.*

hawaiiensis and 63 to 106 days for *L. concolor* (Radtke et al. 1988, 2001). It seems reasonable to suppose that the length of these larval durations is sufficiently long to disperse among the Hawaiian Islands. Dawson et al. (2002) suggested that *C. ios* has a greater dispersal ability in the California transition zone than its sister taxon *E. newberryi* based on habitat structure and life-history differences such as larval duration, which is 2 to 4 weeks in *C. ios* and is possibly as little as a few days in *E. newberryi*. Therefore, it is possible that the duration of the marine larval stage of gobies may be roughly correlated and may be proof of an adaptability to long oceanic transportation.

This suggests that species such as *C. ios*, the Hawaiian amphidromous gobies and *S. japonicus* that may have single panmictic populations are able to disperse over long distances in the sea (Dôtu and Mito 1995, Radtke et al. 1988, 2001, Dawson et al. 2002). For *S. japonicus* it may be easy for their larvae to have a large-scale dispersion because the Kuroshio Current passes by almost all of its species range. A wide geographic distribution of other members of the Sicydiinae also may be supported by a high dispersal ability during their larval periods. However, in the case of *S. japonicus*, there is a need for further investigation of this hypothesis using otolith and mtDNA of additional specimens from Taiwan (located at the southern extreme of the range of this species) and using more rapidly evolving genetic markers such as microsatellite DNA, Amplified Fragment Length Polymorphism (AFLP) and Single Nucleotide Polymorphisms (SNPs).

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