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Genetic Differentiation of the Gobies *Gymnogobius castaneus* and *G. taranetzi* in the Region Surrounding the Sea of Japan as Inferred from a Mitochondrial Gene Genealogy

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ABSTRACT—The phylogenetic relationships between gobies of the genus *Gymnogobius* were analyzed using mitochondrial cytochrome *b* gene sequences, focusing on the species currently classified as *G. taranetzi* and *G. castaneus* that occur in Japan, South Korea, and Russia. Gobies of the two species collected at 12 localities in Japan, South Korea, and Russia formed a monophyletic clade (called the "*castaneus* species complex" here) with *G. breunigii* as the sister clade. Within the species complex, six lineages were recognized: (L1) *G. castaneus* from the Akigawa River, Tokyo, Japan; (L2) *G. castaneus* from Yuza, Yamagata, Japan; (L3) *G. taranetzi* from Russia and South Korea; (L4) *G. castaneus* from the Tonegawa River, Chiba, Japan; (L5a) *G. taranetzi* from Shimane, Japan; and (L5b) *G. castaneus* + *G. taranetzi* from the Japan Sea coast of northern Japan. The two local lineages of *G. castaneus* (L1 and L2) are highly divergent from the others. The Japanese populations of *G. taranetzi* have diverged from the continental *G. taranetzi* populations, while one mitochondrial lineage (L5b) is shared with *G. castaneus* of northeast Japan. Therefore, the current species *G. taranetzi* and *G. castaneus* as defined morphologically are polyphyletic, necessitating a taxonomic revision. The genetic differentiation of isolated local lineages and the evolution of *taranetzi*- and *castaneus*-type gobies have likely occurred repeatedly in brackish/freshwater habitats around the Sea of Japan. We discussed the time of divergence for these gobies based on a tree with the molecular clock assumption.

Key words: biogeography, molecular clock, freshwater fish, mitochondrial DNA

INTRODUCTION

The gobies of the genus *Gymnogobius* (Pisces: Gobiidae) in East Asia are adapted to shallow marine, brackish, and freshwater habitats and were classified into 13 species in a recent taxonomic review (Stevenson, 2002). There are six freshwater species in this genus; three of these (*G. opperiens*, *G. petschiliensis*, and *G. urotaenia*) spend part of their life in marine habitats and do not show any apparent geographical differentiation between Japan and Korea or within these regions (Harada *et al*., 2002). Of the other three species, *G. isaza* is endemic to Lake Biwa (Shiga Prefecture, Japan), whereas *G. castaneus* occurs widely in northern Japan (north from the Kanto and Hokuriku Districts of Japan); *G. taranetzi* occurs along the southern part of the Japan Sea coast from Toyama to Shimane, Japan, and along the coasts of Korea and Russia (Akihito *et al*., 2002; Stevenson, 2002). The latter three species complete their life cycles in freshwater (Kawanabe *et al*., 2001). Aizawa *et al*. (1994) studied the phylogeny of these species based on allozyme polymorphisms and found that *G. castaneous* pop-

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ulations consisted of two highly diverged lineages, one of which included *G. tranetzi* in Japan (so-called "Shinjiko-haze"). Moreover, the divergence between these *G. castaneus* lineages were as large as that for each of them with *G. breuningii* (note changes in scientific names; *Chaenogobius castaneus*, *C*. sp from L. Shinjiko, and *C*. *laevis* in Aizawa *et al*. [1994] are equivalent to *Gymnogobius breunigii*, *G. taranetzi*, and *G. castaneus*, respectively, according to Stevenson, 2002). The existence of the two diverged lineages in *G. castaneus* lineages has been confirmed in an extensive study of allozyme polymorphisms (T. Shinozaki, M. Hatsumi, K. Wakahama, A. Goto, unpublished). However, the phylogenetic relationships among *G. breunigii*, *G. castaneous* and *G. taranetzi* remain to be resolved with samples including continental *G. taranetzi*. In addition, phylogenetic analysis using mitochondrial DNA sequences would be useful to resolve the relationship among the *Gymnogobius* species in comparison with the previous results from the allozyme data.

In this study, we analyzed partial nucleotide sequences of the mitochondrial cytochrome *b* gene in *G. taranetzi* from Primorsky, Korea, and Japan, and in some populations of *G. castaneus* in Japan. Our study demonstrates that the mitochondrial gene genealogy of *G. taranetzi* and *G. castaneus* does not reveal reciprocal monophyly; the continental and Japanese populations of *G. taranetzi* do not have a sister relationship; and the Japanese populations currently classified as *G. taranetzi* and *G. castaneus* likely consist of several distinct lineages or species.

MATERIALS AND METHODS

Sampling and DNA sequencing

The samples used in this study are listed in Table 1. Skeletal muscles were cut from fresh fish or ethanol-fixed specimens and digested in CTAB buffer with proteinase K. Total genomic DNA was extracted using the standard phenol-chloroform method. For PCR amplification of the mitochondrial cytochrome *b* gene region, the primers L15172 (5'-TGA GGA CAA ATA TCN TTY TGA GG-3') and H15915 (5'-A CCT CCG ATC YCG GAT TAC A AG AC-3') were used (Harada *et al*., 2002). In direct sequencing with the PCR products, the dye-terminator, cycle-sequencing reaction was performed with an ABI PRISM BigDye Terminator Cycle Sequencing FS Ready Reaction Kit and reaction products were electrophoresed on an ABI 377 sequencer (Applied Biosystems). The 704-bp sequences are deposited in GenBank (accession: AY450363-

Table 1. List of samples used for the analysis of mitochondrial DNA sequences.

Species [Japanese name] Locality [locality code for Fig. 1]	No. sequenced	No. haplotypes
Gymnogobius castaneus (O'Shaughnessy, 1875) [Juzukakehaze]		
Yoneshirogawa R., Akita, Japan [C1]	1	1
Fukushimagata, Toyosaka, Niigata, Japan [C2]	2	2
Yuza, Yamagata, Japan [C3]	3	1
Tonegawa R., Noda, Chiba, Japan [C4]	3	2
Akigawa R., Tokyo, Japan [C5]	3	1
G. taranetzi (Pinchuk, 1978) [Shinjikohaze]		
Artemovka R., near Vladivostok, Russia [T1]	3	1
Uljin-gun, Gyeongsangbuk-do, South Korea [T2]	3	1
L. Shinjiko, Shimane, Japan [T3]	3	2
Ohtarai-ike, Masuda, Shimane, Japan [T4]	4	1
Maogawa R., Himi, Toyama, Japan [T5]	1	1
Sugatagawa R., Himi, Toyama, Japan [T6]	3	1
Shokawa R., Shinminato, Toyama, Japan [T7]	1	1
G. breunigii (Steindachner, 1880) [Biringo]		
L. Nakaumi, Shimane, Japan	3	3
Maruyamagawa R., Hyogo, Japan	3	2
G. heptacanthus (Hilgendorf, 1879) [Nikuhaze]		
L. Nakaumi, Shimane, Japan	3	3
G. petschiliensis (Rendahl, 1924) [Sumi-ukigori]		
Sugatagawa R., Himi, Toyama, Japan	1	1
G. opperiens Stevenson, 2002 [Shima-ukigori]		
Sugatagawa R., Himi, Toyama, Japan	1	1
G. urotaenia (Hilgendorf, 1879) [Ukigori]		
Uljin-gun, Gyeongsangnam-do, South Korea	3	3

AY450388; AY461730- AY461731).

Phylogenetic analysis

For phylogenetic reconstruction, we used six cytochrome *b* gene sequences of *G. urotaenia*, *G. petschiliensis*, *G. isaza*, *G. opperiens*, *G. uchidai*, and *G. macrognathos* reported in Harada *et al*. (2002) in addition to our data. A sequence of *Rhinogobius giurinus* (Rutter, 1897) was used as the outgroup of *Gymnogobius* (GenBank accession: AB018997; Kumazawa *et al*., 1999). We performed equal-weight parsimony analysis using PAUP* version 4.0b10 (Swofford, 2002) with a heuristic search of 100 random addition analyses with tree bisection-reconnection (TBR) branch swapping (MULTREE option in effect). Support for nodes was assessed using 1,000 bootstrap resamplings and a branch support (Bremer, 1994). TreeRot. v2 (Sorenson, 1999) was used to calculate the branch supports.

Estimation of times of divergence

To estimate the age of divergence between lineages, we compared maximum likelihood trees with and without the molecular-clock assumption using the likelihood ratio test. The maximum likelihood (ML) analysis used PAUP*, and a tree search was performed using a heuristic search of 10 random addition analyses with TBR branch swapping (MULTREE option in effect). The parameters used in the ML analysis followed the results of the hierarchical likelihood ratio test implemented in Modeltest version 3.06 (Posada and Crandall, 1998). The Tamura and Nei (1993) model with the gamma shape parameter was selected as the fittest model. When the ML trees with and without the molecular clock assumption did not differ, we used the former ultrametric tree to estimate age.

RESULTS

Partial nucleotide sequences of the mitochondrial cytochrome *b* gene were determined for 44 specimens (Table 1), and 28 haplotypes were distinguished. A total of 34 haplotypes from 10 species of *Gymnogobius*, including six haplotypes by Harada *et al*. (2002), were used in the phylo-

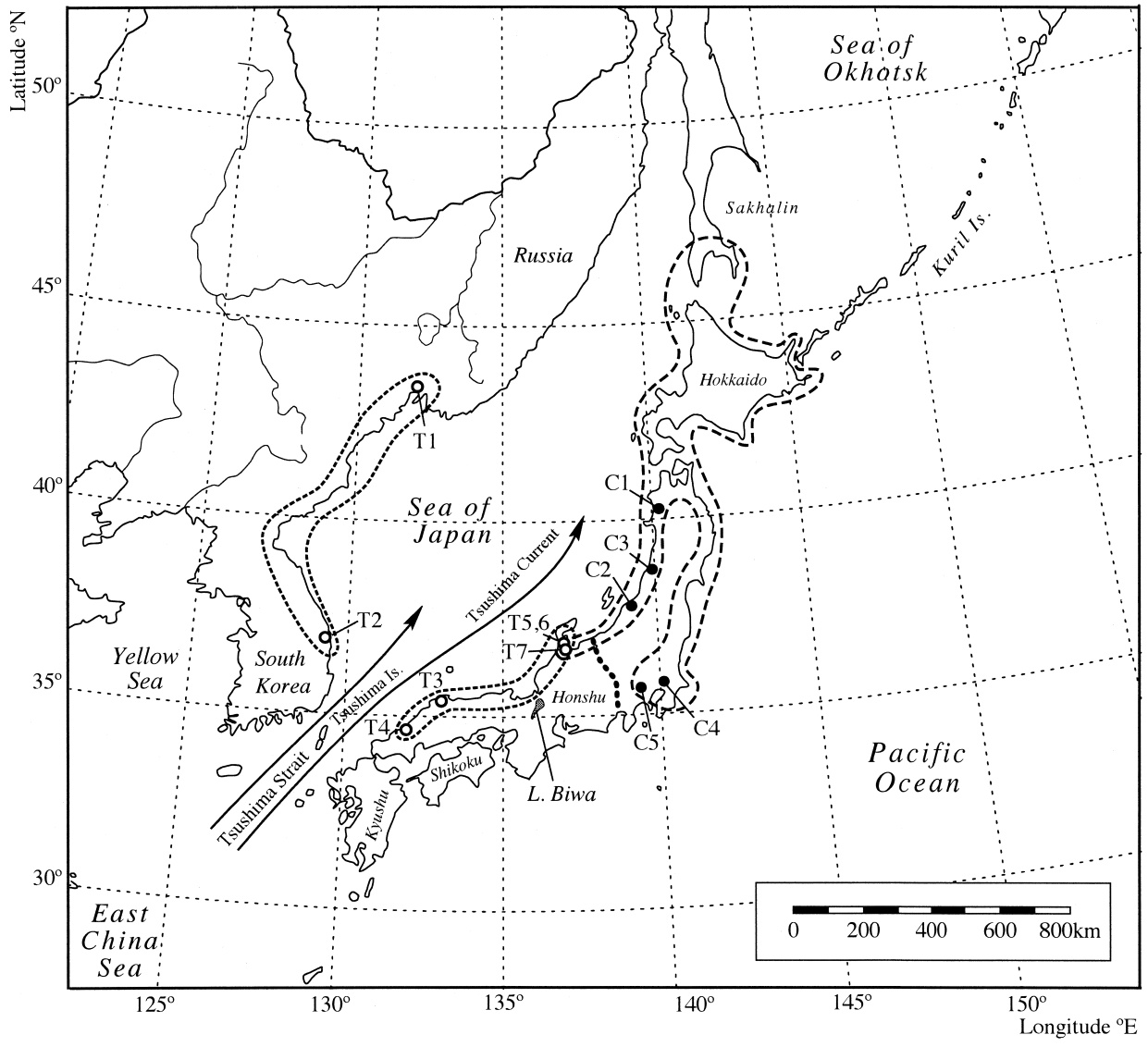

Fig. 1. Map of Japan and Far East, showing sample localities of *Gymnogobius castaneus* (closed circles) and *G. taranetzi* (open circles). Broken lines indicate distribution ranges of *G. castaneus* and *G. taranetzi* (Akihito *et al*., 2002). For locality codes (C1–5; T1–7), see Table 1. The dotted line represents the west margin of Fossa Magna that may correspond to the position of the east channel (see Fig. 3).

genetic analysis with *Rhinogobius giurinus* as the outgroup. The maximum parsimony analysis resulted in four shortest trees of 741 steps (consistency index, CI, excluding uninformative characters=0.50; retention index, RI=0.81; rescaled consistency index, RC=0.42). Most of the branches are supported with high bootstrap percentages, but basal relationships of *Gymnogobius* species were not resolved (Fig. 2).

The *G. castaneus* and *G. taranetzi* sequences formed a monophyletic clade, with *G. breunigii* as the sister clade. The *G. castaneus* and *G. taranetzi* sequences did not reveal reciprocal monophyly. Therefore, we called the monophyletic clade consisting of these two species "the *castaneus* species complex." In this clade, at least five distinct lineages

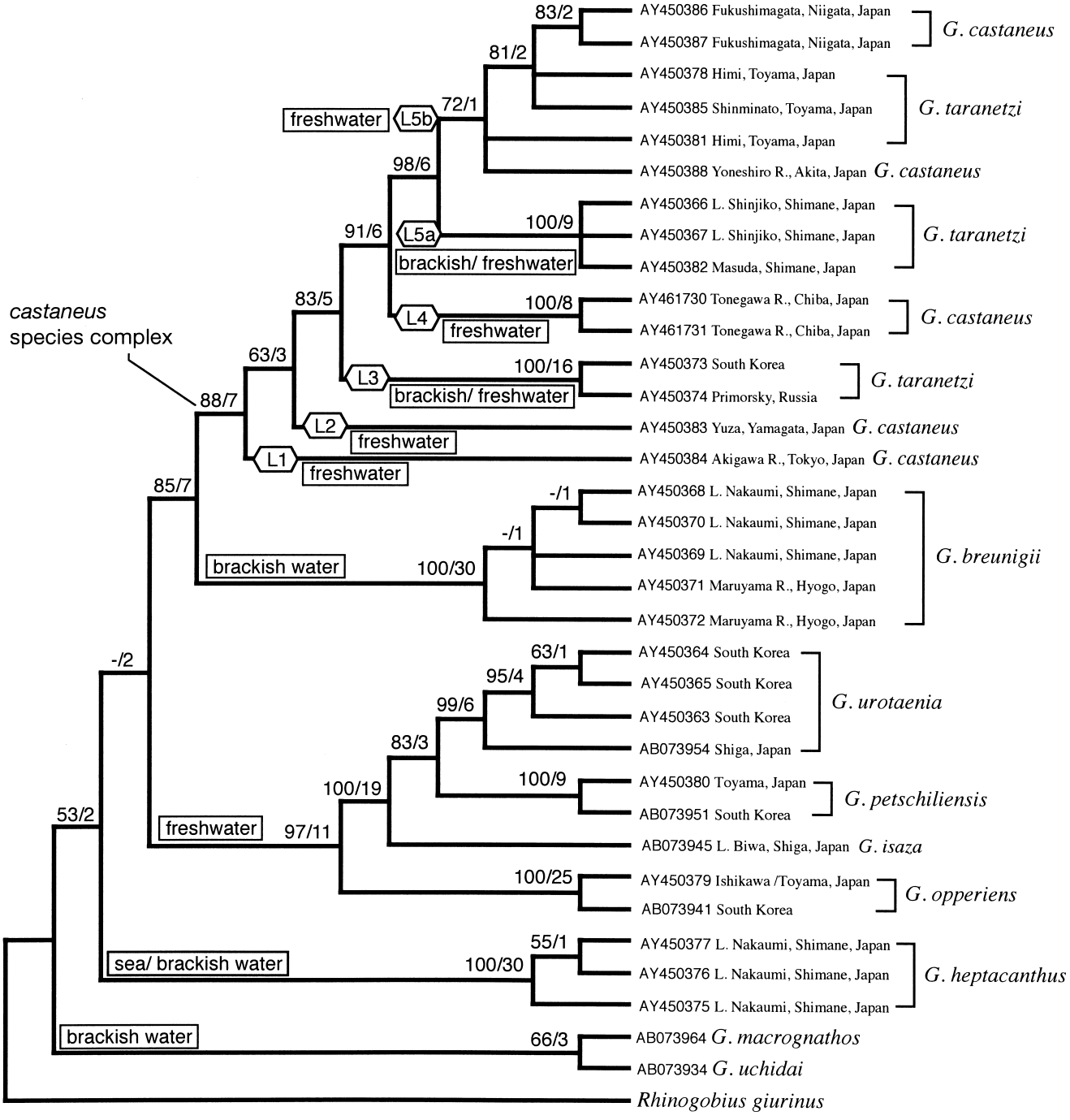

Fig. 2. Strict consensus of four shortest trees resulting from the maximum-parsimony analysis. Bootstrap percentages (when >50%) and branch supports (following "/"; Bremer, 1994) are indicated above the branches. Aquatic habitats (sea, brachkish, and freshwater) are also indicated. GenBank accessions are given at terminals where sequences with AB-numbers are published by Harada *et al.* (2002) and those with AY-numbers by the present study.

are recognized: (L1) *G. castaneus* from the Akigawa River, Tokyo; (L2) *G. castaneus* from Yuza, Yamagata; (L3) *G. taranetzi* from Russia and Korea; (L4) *G. castaneus* from the Tonegawa River, Chiba; and (L5) *G. castaneus* and *G. taranetzi* from the Japan Sea coast of Japan. The last lineage is subdivided into (L5a) *G. taranetzi* from Shimane and (L5b) *G. castaneus* with *G. taranetzi* from the coastal region of northern Japan facing the Sea of Japan.

The likelihood ratio test showed that the ML trees with and without the molecular-clock assumption did not differ significantly ($2\Delta = 2[-4110.5 - [-4127.5]] = 34.0$, df= 34, $P > 0.1$). Therefore, we used the ultrametric tree to estimate the age of divergence of the lineages (Fig. 3). The topology of this tree generally coincided with that of the maximum parsimony tree except for the placement of *G. heptacanthus*. To calibrate the ages, we assumed that the node height (0.047) at the branch between the continental *G. taranetzi* (L3) and the Japanese *G. castaneus-taranetzi* (L4-L5a-b) lineages corresponds to 3.5 million years ago (Ma), which is when the Tsushima Current began to flow into the Sea of Japan (Tada, 1994). Earlier than 3.5 Ma (beginning at about 10.5 Ma), the Tsushima Strait was closed and there was a land bridge between southwest Japan and Korea, enabling the possible migration of gobies across the Sea of Japan; more recently than 3.5 Ma, migration might have been difficult because of the flow of the Tsushima Current. This calibration resulted in a substitution rate of 2.7% per million years (My). With this calibration, the differentiation between the *castaneus* species complex and *G. breunigii* occurred during the mid Miocene (10 Ma). The differentiation of the two Japanese endemic lineages (L1 and L2–L5) has occurred since the beginning of the Pliocene (6 Ma),

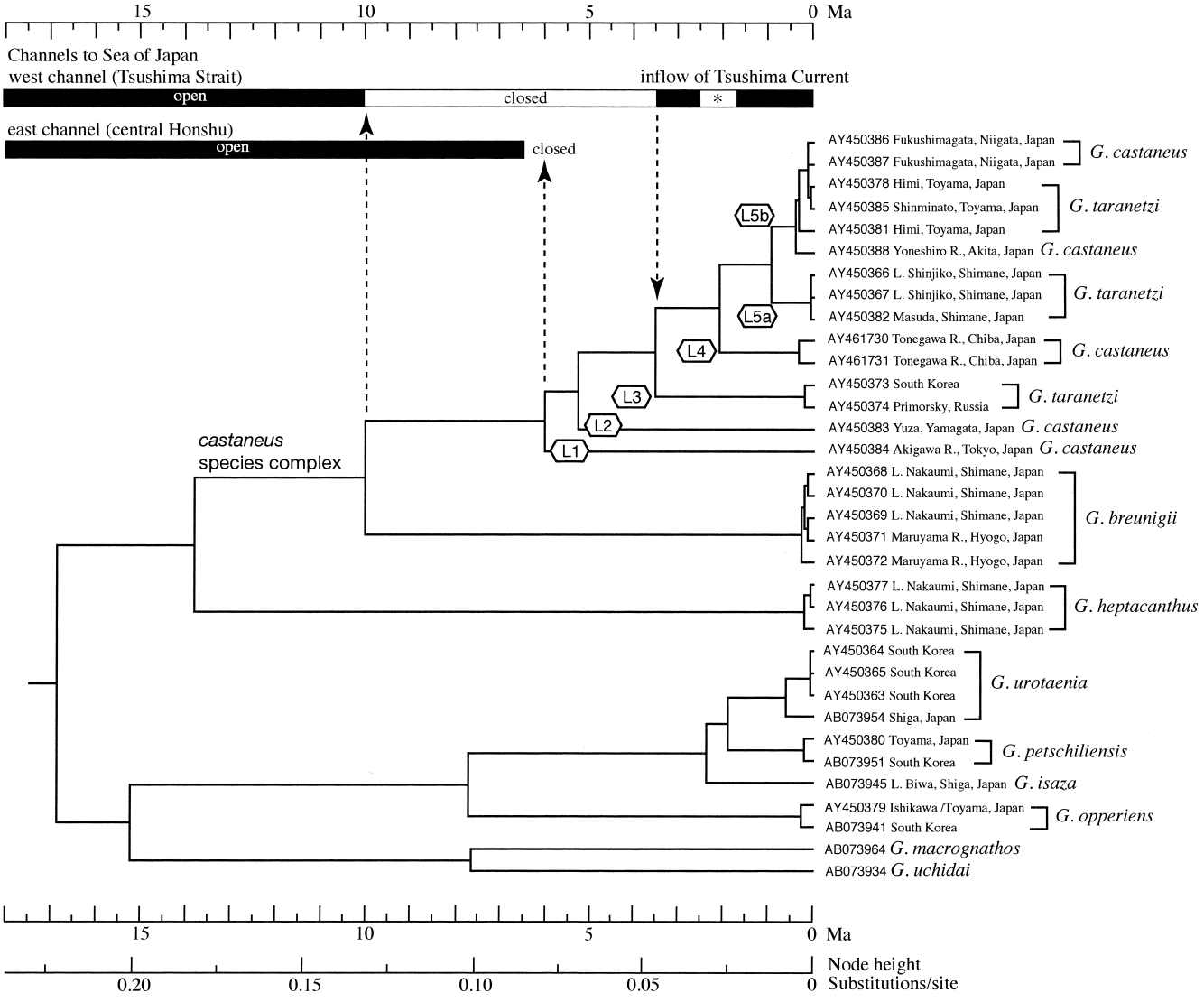

Fig. 3. The maximum likelihood tree with molecular-clock assumption with a calibration of age (3.5 Ma) at the branching of the continental (L3) and Japanese lineages (L4–5a,b). Open (black bar) and closure (open bar) of the west and east channels to the Sea of Japan followed Tada (1994); the closure in 2.5–1.7 Ma (*) of the west channel followed Kitamura *et al.* (2001); note that the west channel might be closed repeatedly during glacial periods after 1.7 Ma.

whereas the differentiation of the other Japanese lineages (L4 and L5a-b) has occurred since the end of the Pliocene (after 2.1 Ma).

DISCUSSION

This study revealed that the gobies currently classified as *G. taranetzi* and *G. castaneus* form a monophyletic clade sister to *G. breunigii* in the mitochondrial gene genealogy. However, these gobies, defined here as the "*castaneus* species complex", consist of divergent mitochondrial lineages (L1–L5) and that the monophyly of each species was not supported. In the mitochondrial gene genealogy (Figs. 2 and 3), two Japanese *G. castaneus* lineages L1 and L2 differentiated the earliest from the rest of the *castaneus* species complex. The continental *G. taranetzi* (L3) and Japanese *G. taranetzi* + *G. castaneus* (L4, L5a, L5b) lineages are well differentiated, and gene flow across the Sea of Japan might have been absent for a long time. There is some differentiation among *G. castaneus* from the Tonegawa River in Chiba (L4), *G. taranetzi* from Shimane (L5a), and *G. castaneus* with *G. taranetzi* from the Japan Sea coast in the northeastern region (L5b). This divergence represents a more recent geographical differentiation within the lineages of *G. castaneus* and *G. taranetzi* in Japan.

The diagnostic characteristic of *G. taranetzi* and *G. castaneus* is the presence/absence of oculoscapular canals and pores (Stevenson, 2002), and our species identification relies on this character. However, our mitochondrial gene genealogy suggests that the character state changed repeatedly within the *castaneus* species complex. Because the oculoscapular canals and pores are present in *G. breunigii*, a brackish species sister to the *castaneus* species complex, loss of these canals and pores (*i.e.*, transformation to the *G. castaneus* type) might have occurred repeatedly in the *castaneus* species complex. Therefore, the previous diagnostic characteristic likely represents homoplasy, and a more detailed morphological analysis is needed to clarify the species status of different populations in the *castaneus* species complex.

The sharing of a mitochondrial lineage (lineage L5b) between *G. castaneus* and *G. taranetzi* from northeastern Japan was unexpected. This suggests that they are in fact two forms of the same biological species, or that, even if they are separate species, they hybridize at the contact zone and mtDNA might have introgressed between the two species (note that all the specimens of *G. taranetzi* in L5b were from the boundary area between the two species; Fig. 1). Further morphological analysis and phylogenetic study using nuclear DNA sequences are necessary. A freshwater fish, the medaka *Oryzias latipes*, has a contact zone between two genetically divergent forms in the coastal area of the Japan Sea (north of Kyoto and Hyogo Prefecture) (Sakaizumi, 1984). In these freshwater fishes, past geographical isolation and secondary contact between northern and southern populations along the Japan Sea coast might have affected the genetic constitution of local populations.

It is intriguing to explore the time and associated geographical setting for the diversification of *Gymnogobius* species in the evolutionary study of freshwater fishes in Japan. However, no fossil *Gymnogobius* is available to identify the past fauna or to calibrate the molecular clock of the mitochondrial genes. In this study, we assumed that the vicariance of the continental and Japanese *G. taranetzi* occurred after 3.5 Ma. This assumption was based on palaeo-oceanographic studies of the Sea of Japan, which indicate that the Tsushima Strait in the southwest of the Sea of Japan (Fig. 1) was closed from 10.5 to 3.5 Ma (Tada, 1994). During this period, dispersal of the *castaneus* species complex might have been possible between the Korean Peninsula and southwest Japan. Since 3.5 Ma, the Tsushima Current has flowed into the Sea of Japan during the interglacial periods. Therefore, population exchange between the continental and Japanese lineages was likely limited after 3.5 Ma. With our calibration method, 3.5 million years corresponds to 9.4% sequence difference, giving a molecular clock of 2.7% per My. This rate is comparable to the previous estimate for *Gymnogobius*, 2.2–2.4% per My, by Harada *et al*. (2002); their estimate was based on the postulation that *G. isaza*, an endemic species in Lake Biwa, originated in ancient Lake Kouga in 2.7–2.5 Ma (Kawabe, 1996) and the range of previously published substitution rates in fish cytochrome *b*, between 0.8% per My (Cantatore *et al*., 1994) and 2.8% per My (Orti *et al*., 1994). In a palaeo-oceanographic study after Tada (1994), Kitamura *et al*. (2001) proposed that the Tsushima Strait was closed again from 2.5 to 1.71 Ma. If we use 1.71 Ma, instead of 3.5 Ma, as the time of divergence between the continental and Japanese lineages of the *castaneus* species complex, the corresponding molecular clock would be unusually fast, 5.5% per My. Therefore, it would be reasonable to assume that the closure of the strait in 2.5-1.71 Ma did not facilitate gene flow between the continental and Japanese populations because of its relatively short duration and/or the effect of the glacio-eustatic sea level fluctuation since 2.5 Ma.

The estimated times of cladistic events in *Gymnogobius* coincide with those of some geohistorical events (Fig. 3). The split between *G. breunigii* and the *castaneus* species complex occurred 10 Ma, the time when the Tsushima Strait was once closed in the late Miocene. The split of L1 (*G. castaneus* from Tokyo) from other populations of the *castaneus* complex occurred 6 Ma, followed by the split of L2 (*G. castaneous* from Yamagata). In the Late Miocene, the Japanese Islands were extensively uplifted (Iijima and Tada, 1990; Yonekura *et al*., 2001). In central Honshu, the channel between the Pacific Ocean and the Sea of Japan along Fossa Magna was closed by 6 Ma (Iijima and Tada, 1990; Tada, 1994; see Fig. 3), and this might result in isolation and differentiation of L1 lineage. Also, in the northern Honshu (Tohoku District), many lake basins appeared following the uplift along the Ohu Backbone Range (Iijima and Tada, 1990; Yonekura *et al*., 2001). This might be related to the

isolation and differentiation of L2 lineage.

Our study revealed that the species complex consisting of the nominal species *G. castaneus* and *G. taranetzi* exhibits notable local divergence, as well as incongruence between the morphological species boundary and mitochondrial lineages. A more thorough sampling of local specimens of this species complex is needed to confirm the several different levels of phylogeographic divergence associated with the geohistory of the Sea of Japan and the Japanese Archipelago.

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