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Evolution of a Ureagenic Ability of Japanese *Mugilogobius* Species (Pisces: Gobiidae)

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ABSTRACT— Interspecific differences in the ability to excrete urea in four gobiid fishes, *Mugilogobius abei*, *M. chulae*, *M. sp.* 1 and *M. sp.* 2, in response to elevated external ammonia, were compared and set against the phylogenetic relationships of species, following molecular phylogenetic analyses using a portion of mitochondrially-encoded 12S ribosomal RNA and tRNA-Val genes, which were sequenced from five species of *Mugilogobius*. The resulting tree, based on 710 base pairs of aligned sequences, was statistically robust and indicated two major clades, (*M. abei*, *M.* sp. 1 and *M. chulae*) and (*M. parvus* and *M.* sp. 2), respectively. High ureagenic ability was demonstrated in *M. abei* and *M.* sp. 1, being sister species according to the phylogenetic analysis, while the remaining species having a low level of ability. The two former species are restricted to temperate and subtropical Japan, whereas the others are widely distributed throughout tropical Asia. The most parsimonious reconstruction of these features suggested that ancestral *Mugilogobius*, probably widely distributed in tropical Asia, may had low ureagenic ability. The high ureagenic ability of the temperate species may have evolved concurrently with the northward spread of *Mugilogobius* from the tropical zone.

INTRODUCTION

In non-teleost fishes, the ability to produce urea via the ornithine-urea cycle (OUC) exists and may be important for survival (Griffith, 1991); e.g. chondrichthyes and coelacanths maintain their body fluid osmolarity at a hypertonic level (equal to seawater) by urea accumulation (Anderson, 1980; Brown Jr. and Brown, 1967; Webb and Brown Jr., 1980) and ureotely in lung fishes is important for estivation during the dry season (Jannsen, 1964; Smith, 1930).

The majority of teleosts, however, have non-detectable or very low activities of the key enzymes in OUC, in particular carbamoyl phosphate synthetase (CPSIII), which catalyzes the first entry of nitrogen into the cycle. Moreover, in large-mouth bass (*Micropterus salmoides*), CPSIII and CPSIII mRNA occur at low levels and urea production barely increases in response to elevated external ammonia (Kong *et al.*, 1998), i.e. no functional ureagenesis. [The term “ureagenesis” is used in a broad sense, and “ureogenesis” is in narrow sense, which mean only urea synthesis via the OUC (Mommsen and Walsh, 1991).] On the other hand, some teleosts have been reported to have functional ureagenesis in spite of CPSIII activity in the liver being non-detectable (Wright, 1993). In these cases, it has been assumed that urea is produced mainly via the degradation of purine (uricolysis), although a few teleosts are clearly known to produce urea via the OUC (Mommsen and Walsh, 1991).

Although the biochemical mechanisms may vary, being still unclear, several examples of ureagenesis in teleosts have been related to survival strategies in extreme environments. In Lake Magadi, an alkaline soda lake (pH=9.6–10) into which fishes cannot excrete ammonia, only tilapia (*Oreochromis alcalicus grahami*), which performs complete ureogenesis (= ureagenesis via the OUC), occurs (Randall *et al.*, 1989). The air-breathing cat fish (*Heteropneustes fossilis*),which performs functional ureagenesis, inhabiting tropical pools may also be adapted to temporary dehydration or a hyper-ammonia environment (Saha and Ratha, 1987; Saha and Ratha, 1989).

The gobiid fishes, which have undergone vast ecological divergence, comprise more than 200 genera and 1,800 species (Nelson, 1994), including some extremely specialized forms. Although it seems likely that the extremely specialized

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goby show ureagenic ability, even the typically amphibious goby, *Periophthalmus modestus* (mudskipper), did not increase urea production following exposure to either air or high external ammonia (Iwata and Deguchi, 1995). On the other hand, it has been found that *Mugilogobius abei* can produce a large amount of urea, when exposed to high external ammonia although CPSIII activity could not be detected in the liver (Iwata, 1998; Iwata, unpublished data). *Mugilogobius* species inhabit mainly brackish water habitats, ranging from Japan to southern Africa and Oceania (Hoese and Winterbottom, 1979). The Japanese Archipelago represents the northern limit of *Mugilogobius* habitat, 6 species being recognized in Japan (Akihito *et al*., 1993). Of these, *M. abei*, the only species distributed in temperate Japan, is known to undergo apparently functional ureagenesis (Iwata, 1998), the occurrence or otherwise of such in the other 5 species (distributed in subtropical Ryukyu Is.) being to date unknown. Because the geographical distributions and ecological features of the subtropical species differ from each other (Fig. 1), interspecific comparisons of their habitats and physiological characters, especially ureagenesis, are important so as to determine the physiological evolution of *Mugilogobius*.

Interspecific comparisons, indeed any evolutionary or adaptive hypothesis, are best based on an explicit, well-corroborated phylogeny, such being fundamental to an understanding of the evolution of biological diversity, through the light shed on character constraint or convergence.

Molecular phylogenetic methods, for example, allozyme analysis, restriction fragment length polymorphisms and DNA sequence analysis, have been established as effective tools for estimating phylogenetic relationships. Mitochondrial DNA (mtDNA) sequences are particularly useful because of their rapid evolutionary rate and the absence of recombinations (Avise, 1994). Therefore mtDNA sequences are considered to be good molecular markers for the reconstruction of animal phylogenies. Even though mtDNA phylogenies may be powerless in the face of saturation by nucleotide substitutions among highly divergent taxa (Meyer, 1994) or introgressions among a hybridizing species complex (Mukai *et al*., 1997), many successful examples have been reported (Avise, 1994).

In the present study, we determined 710 base pairs (bp) of mtDNA sequences from 12S ribosomal RNA and tRNA-Val

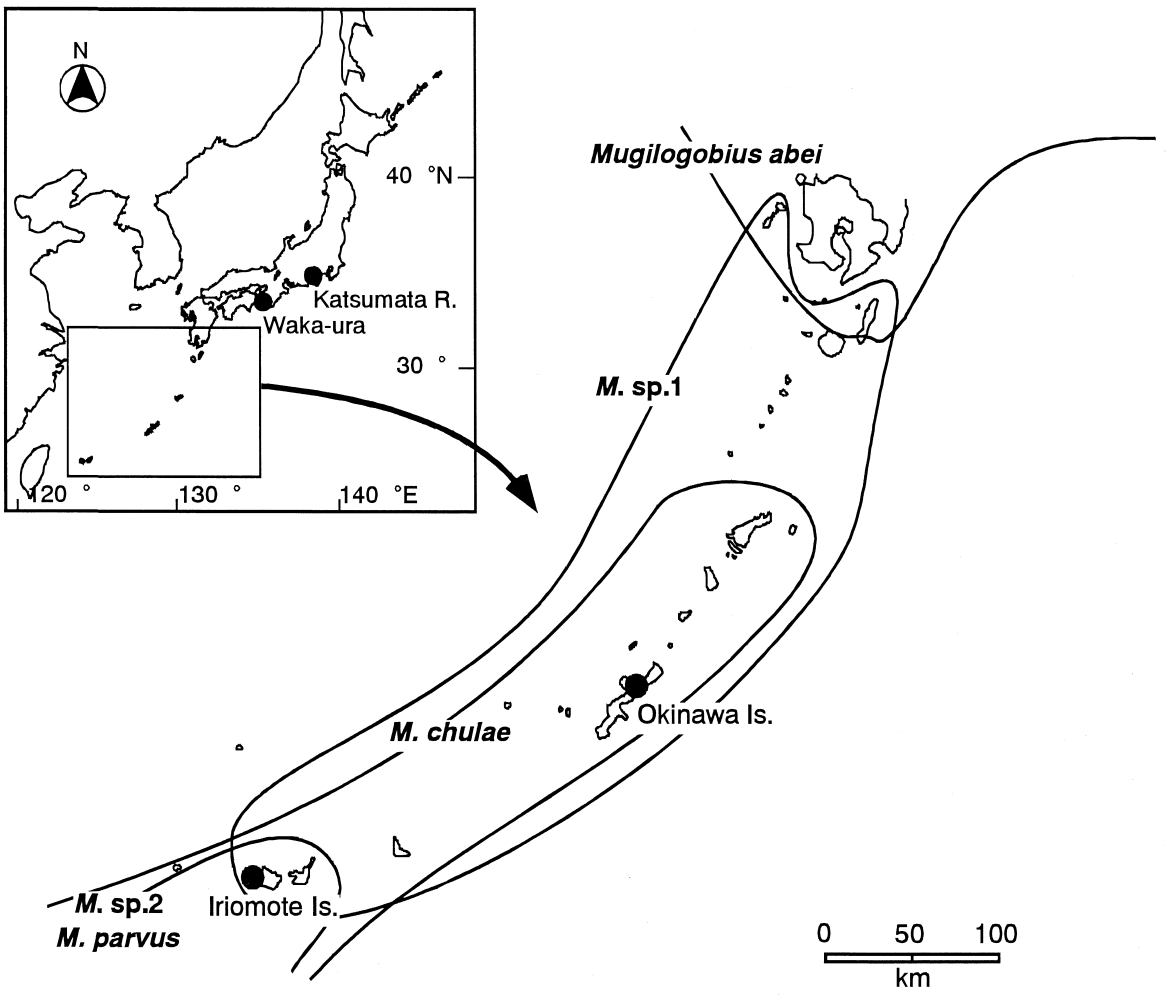

Fig. 1. Geographical distributions of Japanese *Mugilogobius* species (referred from Akihito *et al*., 1993). Solid circles show the sampling sites (Katsumata R., Waka-ura, Okinawa Is. and Iriomote Is.).

genes for 5 species of *Mugilogobius* and two outgroup taxa, and subjected the data to phylogenetic analyses. The topology of the resultant tree was well resolved, enabling the proposal of a hypothesis for the evolution of this physiological character in gobiid fishes.

MATERIALS AND METHODS

Experimental animals

Gobiid fishes were collected from Waka-ura (Wakayama Pref.) (*Mugilogobius abei*), Katsumata River (Shizuoka Pref.) (*M. abei*), Okinawa Is. (*M. chulae* and *M.* sp. 1 (Izumi-haze)) and Iriomote Is. (*M. chulae* and *M.* sp. 2 (Tanuki-haze)) (Fig. 1). Each species was identified after Akihito *et al.* (1993). Of these, *M. abei* from Katsumata River and *M. chulae* from Okinawa Is., were used for only DNA sequencing. The other species, *M. parvus* being rare, could not be used for physiological comparisons, although one specimen from Iriomote Is. was used for DNA sequencing. Although *M. fuscus* had been recorded in Japan (Akihito *et al.*, 1993), this species was too rare and was not collected. Fishes were kept in 20% artificial sea water (SW; Jamarine-U synthetic sea water, Jamarine Laboratory, Japan), under controlled temperature and photoperiod (22±2°C, 12L12D) for 2–4 weeks before experimentation. During this time, they were fed commercially supplied dry food (TetraMin®), although feeding ceased 2–3 days before experimentation.

Experimental protocols

After the rearing period, each individual (weighing 0.14–0.49 g; eight individuals per species) (Table 1) was placed into an experimental vessel with 20–40 ml 20% SW for 3 days. The SW was replaced after each 24hr period, the water replaced being stored at –20°C until analyses. Subsequently, the SW was replaced with 2 mM NH_4HCO_3 in 20% SW for 5 days. This was also replaced after each 24hr period and stored as above. Ammonia and urea concentrations were measured spectrophotometrically following Sorozano (1969) and Ceriotti and Spandrio (1963), respectively. Ammonia excretion was not measured during the ammonia treatment period because of the high concentration of ammonia in the experimental sea water and the uncertain uptake by the fishes. The Mann-Whitney U test was used for statistical analyses.

DNA extraction, amplification and sequencing

Total DNA of *M. abei* , *M. chulae*, *M. parvus* , *M.* sp. 1 (Izumi-haze), *M.* sp. 2 (Tanuki-haze), and two operational outgroup taxa, *Eleotris oxycephala* (Gobioidei: Eleotridae) and *Tridentiger obscurus* (Gobioidei: Gobiidae), was extracted from the liver by proteinase K digestion with TNES-6M urea buffer (10 mM Tris pH7.5, 125 mM NaCl, 10 mM EDTA, 6 M urea, and 1% SDS) (Tewari *et al.*, 1992), phenol/chloroform extraction and ethanol precipitation, and dissolved in 1 mM EDTA buffered with 10 mM Tris at pH 8.0. A partial region of the 12S ribosomal RNA and tRNA-Val gene (about 730 bp) was amplified by the polymerase chain reaction (PCR) (Saiki *et al.*, 1988) using the following primer pair: OMT16SF (5'- TGC CAG CCA CCG CGG TTA TAC CT -3') and tRNA02 (5'- GGA TGT CTT CTC GGT GTA AG -3'). AmpliTaq®DNA polymerase (Perkin Elmer Cetus, U.S.A.) was used for PCR. The temperature regimen for 30 cycles was 1 min at 95°C for denaturation, 1 min at 55°C for annealing and 2 min at 70°C for extension. The amplified products were purified and concentrated by a spin column of UFC3LTK00 (Millipore, Japan). All sequencing reactions were performed according to the manufacture's instructions. Labeled fragments were analyzed on an ABI prism 310 genetic analyzer (Perkin-Elmer). Sequences were obtained from both strands of the gene segments for verification by three forward primers, OMT16SINTF (5'- CTG GGA TTA GAT ACC CCA C -3'), OMT16SR (5'- ACG TCA GGT CGA GGT GTA GCA TAT -3') and valINTF (5'- AAT GTA GCC CAT TTC TTC C -3'), and three reverse primers, MT12SLR (5'- AGG TTT AAC GGG GGT TAT CGG TTC TAG AAC AGG CTC -3'), OMT16SINTR (5'- GTC CTT TGG GTT TTA AGC T -3') and tRNA01 (5'- TGT TAC GAC TTG CCT CCC C -3'). These primers, except tRNA01 and 02, were designed by Dr. K. Naruse (Univ. of Tokyo). All sequences are available from DDBJ under accession numbers AB022892-AB022897 and AB022901.

Phylogenetic analysis

Multiple alignment of sequences was done using the Clustal V program with default settings (Higgins and Sharp, 1988). Computer-generated alignments were compared to secondary-structure models described by Neefs *et al.* (1991) and Ortí *et al.* (1996). Based on these models, the partial ribosomal RNA gene sequences obtained from gobies were improved by eye using manual aligner SeqApp 1.9 (Gilbert, 1993). The PHYLIP ver. 3.572 package (Felsenstein, 1996) and PAUP* 4.0 (beta test version) (Swofford, 1998) were used for the

Table 1. Interspecific differences in the rates of urea and anmonia excretion among 4 species of *Mugilogobius* (N=8 / species).

Species	(Body weight)	20% SW			20% SW+Ammonia				
	(Days)	1	2	3	4	5	6	7	8
Urea excretion: Mean±S.E. (μmol/g/day)									
M. abei	(0.31–0.46g)	4.17±0.30	4.53±0.50	3.24±0.42	4.12±0.53	11.99±1.96	28.90±2.62	29.04±1.89	28.25±2.95
M. sp. 1	(0.14–0.25g)	3.74±0.47	4.17±0.35	3.21±0.40	6.34±0.52	13.11±2.11	28.60±2.07	19.06±4.31	15.49±2.11
M. chulae	(0.31–0.39g)	1.76±0.39	2.30±0.44	1.98±0.27	1.56±0.13	4.30±0.53	7.93±1.72	8.40±1.76	9.17±1.68
M. sp. 2	(0.38–0.49g)	1.53±0.32	1.72±0.29	1.65±0.29	2.69±0.58	2.10±0.37	4.66±1.24	4.11±1.12	3.47±0.77
Ammonia excretion: Mean±S.E. (μmol/g/day)									
M. abei		13.13±1.00	13.83±1.37	11.33±1.07	–	–	–	–	–
M. sp. 1		16.74±1.77	16.80±2.36	13.89±1.70	–	–	–	–	–
M. chulae		8.88±0.85	7.59±1.01	7.92±1.12	–	–	–	–	–
M. sp. 2		7.58±1.77	10.69±1.94	8.58±1.51	–	–	–	–	–
Significant difference in urea excretion									
M. abei vs.*M.* sp. 1		N.S.	N.S.	N.S.	**	N.S.	N.S.	*	**
M. abei vs. *M. chulae*		**	**	*	**	***	***	***	***
M. abei vs. *M.* sp. 2		***	***	*	*	***	***	***	***
M. sp. 1 vs. *M. chulae*		*	*	*	***	*	***	*	*
M. sp. 1 vs. *M.* sp. 2		**	***	*	**	**	***	***	***
M. chulae vs. *M.* sp. 2		N.S.	N.S.	N.S.	N.S.	*	N.S.	N.S.	*

N.S., not significant; *, $P < 0.05$; **,$P < 0.01$; ***,$P < 0.001$.

phylogenetic analyses.

Genetic distances were calculated using the Jukes-Cantor model (Jukes and Cantor, 1969) and Kimura's two parameter (K2) model (Kimura, 1980), and a neighbor-joining trees (Saitou and Nei, 1987) constructed. Criterion to choose the models was followed after Kumar *et al*. (1993). PHYLIP was used for the calculation of K2 distance assuming constant transition/transversion ratio. PAUP* was used to construct neighbor-joining tree using only transition or transversion differences. Maximum parsimony (MP) analyses were performed by PAUP* and MacClade 3.06 (Maddison and Maddison 1996). All phylogenetically uninformative sites were neglected in MP analyses. The maximum likelihood tree (Hasegawa *et al*., 1985) was also searched by PAUP*. To evaluate the robustness of the internal branches of the MP and ML trees, 1,000 bootstrap pseudoreplications (Felsenstein, 1985) were executed, with the branch- and -bound algorithm being performed at each replication.

RESULTS

Interspecific differences in ammonia and urea excretion

When exposed to 2mM NH_4HCO_3, the urea excretion increased markedly in *Mugilogobius abei* and *M*. sp. 1 (Fig. 2 and Table 1). In *M. abei*, the mean value of the three days before ammonia exposure was 3.98±0.25 (mean±S.E., μmol/g/day). The urea excretion increased significantly after the 5th day (the 2nd day of exposure), reached 28.90±2.62 in the 6th day and became stable. The urea excretion in *M* sp. 1 also increased from 3.71±0.24 (mean value of the days before exposure) to 28.60±2.07 (the 6th day), those after the 7th day decreased to 15.49±2.11 (the 8th day) (P<0.05, the 6th day vs. the 8th day).

Urea excretion in *M. chulae* on the 5th day (the 2nd day of exposure) also increased to 4.30±0.53 (the 5th day), which were significantly (P<0.001) higher than those of the mean value before ammonia exposure (2.01±0.21), and reached 9.17±1.68 (the 8th day). By contrast, those in *M*. sp. 2 increased significantly (P<0.05) only in the 6th and 7th days (the 3rd and 4th days of exposure). The highest value in *M*. sp. 2 was 4.66±1.24 (the 6th day), which was close to the rate of urea excretion in *M. abei* before ammonia exposure, though mean value in *M*. sp. 2 before exposure (1.63±0.17) was lower than that of *M. abei*.

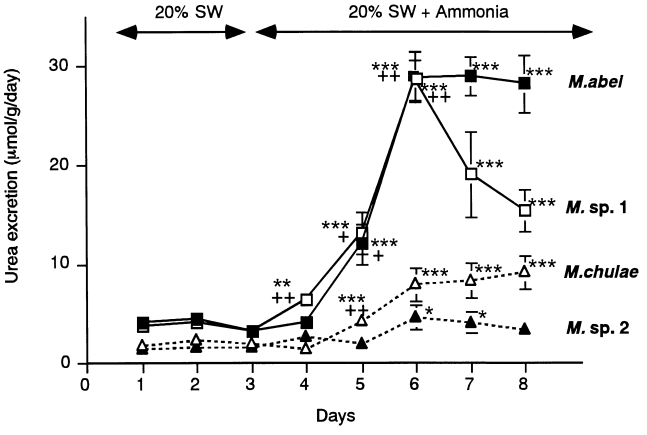

Fig. 2. Interspecific differences in the rates of urea excretion among 4 species of *Mugilogobius* (N=8/species). Bars indicate standard errors (>±1.0). *, P<0.05; **, P<0.01; ***, P<0.001 (vs. mean value of the three days before ammonia exposure). +, P<0.05; ++, P<0.01 (vs. value of the previous day).

As mentioned above, the interspecific differences in the urea excretion were quite significant (Table 1). The rates of urea excretion in *M. abei* and *M*. sp. 1 were significantly higher than in *M. chulae* and *M*. sp. 2 at all times (Fig. 2 and Table 1). Although those in *M. abei* and *M*. sp. 1 were similar (Fig. 2), significant differences were observed in the 4th (P<0.01), 7th (P<0.05) and 8th (P<0.01) days (the 2nd, 4th and 5th days of exposure). The interspecific differences between *M. chulae* and *M*. sp. 2 were less obvious, and significant differences were observed only in the 5th (P<0.05) and 8th (P<0.05) days (the 2nd and 5th days of exposure).

The rates of ammonia excretion before the ammonia exposure were 12.76±0.68 (mean±S.E., μmol/g/day) in *M. abei* (N=8×3 days), 15.81±1.12 in *M*. sp. 1 (N=8×3 days), 8.13±0.56 in *M. chulae* (N=8×3 days) and 8.95±1.00 in *M*. sp. 2 (N=8×3 days). These were higher than urea excretion (<4 μmol/g/day) during the same period.

Mitochondrial DNA sequence variations

Multiple sequence alignment of the amplified region of the 12S ribosomal RNA (rRNA) and tRNA-Val genes from the 5 species of *Mugilogobius* and two outgroup taxa resulted in a matrix consisting of 710 positions, owing to inferred insertion/deletion events (Appendix 1). Fragment sizes from individual taxa ranged from 701–708 base pairs (bp). In the ingroup, fragment sizes of *M. abei*, *M*. sp. 1 and *M. chulae* were 702 bp, and those of *M*. sp. 2 and *M. parvus* 701 bp. The intraspecific sequence differences were very few: two sequences of *M. abei* (collected from Wakayama and Shizuoka) were exactly the same, and two sequences of *M. chulae* (collected from Okinawa and Iriomote Is.) showed only one transition difference at the position number 206 (Appendix 1). Base frequencies of 8 sequences were 0.34±0.01 (A), 0.25±0.01 (C), 0.21±0.01 (G) and 0.19±0.01 (T) (Mean±S.D.), and (A+T)/(G+C) ratios (1.15±0.05) were not so biased.

Of the 710 bp aligned sequences, 180 sites (25.4%) were variant. In the interspecific comparisons, the number of transitions (TS) and transversions (TV) ranged from 13–72 and 2–49, respectively, and the ratios of TS/TV from 1.25 (*Eleotris oxycephala* vs. *Tridentiger obscurus*) to 6.50 (*M. abei* vs. *M*. sp. 1).

Phylogenetic analyses

Figure 3 shows the neighbor-joining (NJ) tree based on Kimura's two parameter model (TS/TV=2.0). Although genetic distances were also estimated using the Jukes-Cantor model, the topology of the resulting NJ tree did not differ from that constructed by Kimura's model. The NJ tree using only transition or transversion differences was also the same topology. The tree indicated that lineages of the mtDNA of *Mugilogobius* belong to either of two major clades, one composed of *M. abei*, *M*. sp. 1 and *M. chulae* (bootstrap probability: BP=96%) and the other of *M. parvus* and *M*. sp. 2 (BP=95%). In the

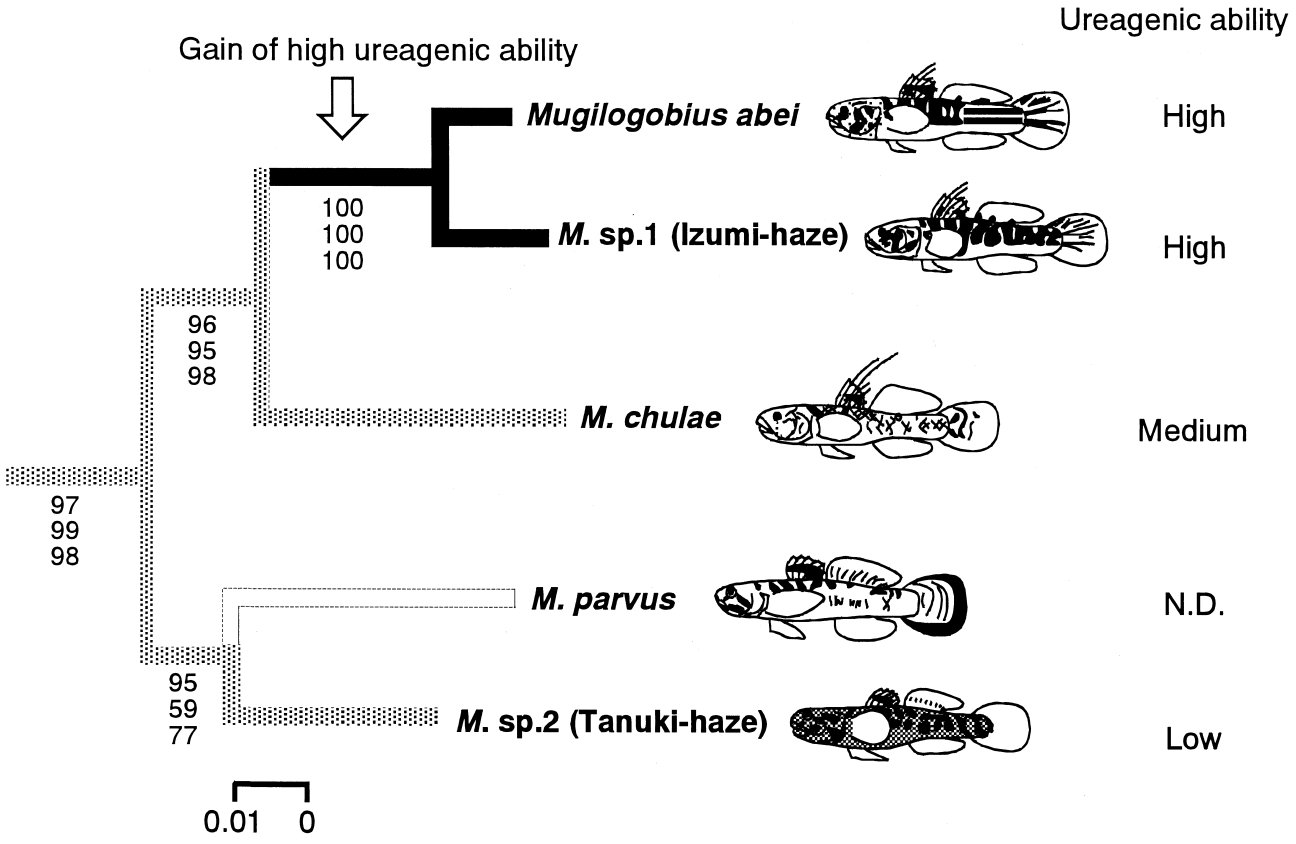

Fig. 3. Neighbor-joining tree based on genetic distances estimated from partial mitochondrial 12S rRNA and tRNA-Val gene sequences of *Mugilogobius*. The sequence for *M. chulae* was based on a specimen from Iriomote Island. Distances were estimated by Kimura's two parameter model (TS/TV=2.0). Scale indicates genetic distance. Fish illustrations are modified from Akihito *et al.* (1993). Numbers beside internal branches indicate bootstrap probabilities of NJ (above), MP (middle) and ML (below) trees, based on 1,000 pseudoreplicates.

former clade, *M. abei* and *M.* sp. 1 comprised a monophyletic group, supported by a high bootstrap probability (BP=100%).

Parsimony analysis, using an exhaustive search option (gaps treated as missing data, all characters unordered and equally weighted), resulted in a most parsimonious (MP) tree of 945 trees consistent with the NJ tree. The statistical values of the MP tree were: tree length=270, consistency index=0.82, consistency index excluding uninformative characters=0.73, retention index=0.67 and rescaled consistency index=0.55. The monophyly of *M. abei* and *M.* sp. 1 was also supported by a high bootstrap probability (BP=100%). The two major clades were supported statistically by 97 and 59% bootstrap probabilities, respectively (Fig. 3). Although the monophyly of *M. parvus* and *M.* sp. 2 decayed in the second shortest tree (tree length=271), the tree length differences between the MP and second most parsimonious trees increased from one to 5 (TS1/TV2) and 13 (TS1/TV4) when the transition / transversion weighting schemes were used (TS1/TV2 and TS1/TV4: numerals indicate weights for transition and transversion). The tree lengths of MP trees with weighting schemes were 356 (TS1/TV2) and 528 (TS1/TV4), and for the second shortest trees 361 (TS1/TV2) and 541 (TS1/TV4). Accordingly, the monophyly of *M. parvus* and *M.* sp. 2 was supported by 80% (TS1/TV2) and 92% (TS1/TV4) bootstrap probabilities.

An attempt to find the maximum likelihood (ML) tree using two parameters (TS/TV=2 or 10) resulted in a topology unaffected by the TS/TV ratio, the best ML tree being the same as the NJ tree. Likelihood was calculated by the HKY model (Hasegawa *et al.*, 1985), Ln likelihood being –2274.69 (TS/TV=2) and –2324.50 (TS/TV=10). Although the bootstrap probability, which supported the monophyly of *M. parvus* and *M.* sp. 2, was also low (77%) for the assumption TS/TV=2 (Fig. 3), the probability increased to 90% for the assumption TS/TV =10.

DISCUSSION

Phylogeny of Japanese *Mugilogobius*

The phylogenetic tree resulting from the mitochondrial DNA (mtDNA) analysis of the 5 species of *Mugilogobius* was statistically robust, the three different algorithms (neighbor-joining, maximum parsimony and maximum likelihood), with various parameter settings indicating a single tree topology. Although the monophyly of the two major clades was not supported by high bootstrap probabilities in the parsimony analysis, the weights for transversions (TV) dramatically increased their robustness. It was likely that the low bootstrap probabilities of the internal branches were caused by multiple transition (TS) hits, since TS differences are known to accumulate rapidly (Meyer, 1994). Thus, although the statistical values

may have decreased owing to multiple substitutions, the mtDNA phylogeny of the Japanese *Mugilogobius* is in no doubt.

Morphological features also support the phylogenetic relationships within *Mugilogobius* (Akihito *et al*., 1993). The first dorsal fin spines were prolonged in *M. abei*, *M.* sp. 1 and *M. chulae*. This character may be a synapomorphy of these three species, because the other *Mugilogobius* species and those in the closely-related genus *Chlamydogobius* do not have prolonged first dorsal fin spines (Miller, 1987). Similarly, *M. abei* and *M.* sp. 1 shared a characteristic color pattern on the caudal fin. Thus, these apparent synapomorphies support 1) *M. abei* and *M.* sp. 1 being sister species, and 2) *M. abei*, *M.* sp. 1 and *M. chulae* comprising a monophyletic group. Although about 30 nominal forms (≠species) belonging to *Mugilogobius* exist in the Indo-Pacific and Oceania (Hoese and Winterbottom, 1979), the sister relationships of *M. abei* and *M.* sp. 1 may not decay because their diagnostic color patterns do not shared with the others.

Evolution of ureagenic ability

When exposed to high ambient ammonia, the 4 species of *Mugilogobius* can be divided into two groups, according to differences in their ability to produce urea (Fig. 2 and Table 1). One group consisted of *M. abei* and *M.* sp. 1, and the other, *M. chulae* and *M.* sp. 2. The habitats of the former group, *M. abei* and *M.* sp. 1, is specialized, and both species are able to survive in eutrophicated muddy environments, in which other fishes can not (Iwata, 1989). On the other hand, the latter group had not been found in eutrophicated environments.

The evolution of ureagenic ability was traced on the molecular phylogenetic tree using MacClade ver. 3.06 (Maddison and Maddison, 1996) as the most parsimonious reconstruction set. [Character state of outgroup was assumed to low ureagenic ability because functional ureagenisis has not been known in any other gobiid fishes (Danno and Iwata, unpublished data)]. The result suggested that the ancestral state of *Mugilogobius* probably had low ureagenic ability, because earlier diverged species (*M. chulae* and *M.* sp. 2) had low abilities of urea production. Furthermore, monophyly of *M. abei* and *M.* sp. 1, which had high ureagenic ability and could inhabit in eutrophicated environments, suggested that the high ureagenic ability is likely to have evolved only once, in the common ancestor of *M. abei* and *M.* sp. 1 (Fig. 3). Although ureagenesis in *M. parvus* was not measured, it is likely to also have low ureagenic ability, because *M. parvus* was collected from a clear freshwater stream on Iriomote Island together with many other freshwater fishes (Mukai, unpublished data).

The ability to produce urea in responce to elevated external ammonia also differed between *M. abei* and *M.* sp. 1, the rate of urea production of *M. abei* being the higher of the two. Furthermore, that of *M. chulae* was higher than that of *M.* sp. 2. Thus, ureagenic ability may be ordered from low to high (*M.* sp. 2<*M. chulae* < *M.* sp. 1<*M. abei*), such coinciding with the branching order of the phylogenetic tree (*M.* sp. 2 (*M. chulae* (*M.* sp. 1, *M. abei*))) (Fig. 3). If this association is an accurate representation, functional ureagenesis might have evolved in step with speciation within *Mugilogobius*.

Phylogeography and functional ureagenesis

Geographic distributions of the Japanese *Mugilogobius* species are differrent (Akihito *et al*., 1993) (Fig. 1). Two species (*M. parvus* and *M.* sp. 2) are restricted to Ishigaki, Iriomote and tropical Asia, whereas *M. chulae*, which has a more northern limit than *M. parvus* and *M.* sp. 2, is distributed widely in Amami-Oshima, Okinawa, Ishigaki, Iriomote and the western Pacific. *M.* sp. 1 is restricted to the subtropical Ryukyu Is. and Kyushu, whereas *M. abei* is found only in temperate Japan and at similar latitudes on the Asian mainland. The phylogenetic tree (Fig. 3) also suggests that the ancestral geographic distribution area of Japanese *Mugilogobius* was tropical Asia, because all of the earlier diverged species (*M. chulae*, *M. parvus* and *M.* sp. 2) were widely-distributed in tropical Asia (Fig. 1).

Furthermore, ureagenic ability parallels the geographical distribution from tropical to temperate zones, the tropical species, *M.* sp. 2 (and perhaps *M. parvus*), showing the lowest ureagenic ability, and the tropical to subtropical species, *M. chulae*, showing slightly greater ability. Also subtropical, *M.* sp. 1 had a greater rate of urea production than *M. chulae*, but less than the temperate *M. abei* (Fig. 2 and Table 1).

This allows the proposal of a hypothesis that functional ureagenesis in *Mugilogobius* evolved as an adaptation to a more temperate environment. Although this hypothesis does not indicate any particular selective force, it is a natural conclusion that functional ureagenesis may be related to adaptation because other known ureagenic teleosts, for example, alkaline soda lake tilapia (Randall *et al*., 1989) and air-breathing cat fish (Saha and Ratha, 1987; Saha and Ratha, 1989), inhabit extreme environments. Thus some factors promoting evolution of functional ureagenesis may exist in the environmental gradient from tropical to temperate zones.

The hypothesis tentatively proposed here, is based only on an analysis of the Japanese *Mugilogobius* species. On the other hand, the closely-related genus *Chlamydogobius*, which occurs in arid Australia, is also adapted to extreme environmental conditions: water temperatures ranging from 5–41°C and maximum salinities reaching 60‰ (twice that of sea water) (Glover, 1982). Thus, physiological, ecological and phylogenetic analyses of *Mugilogobius* and closely-related genus should be undertaken, since these may shed much light on certain aspects of adaptive evolution.

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Appendix 1

Aligned DNA sequences from 710 bp of the 12S rRNA and tRNA-Val genes. Identity with first sequence denoted by dots. Insertions/ deletions of specific nucleotides indicated by dashes (–). #1 and #2 are sequences from *M. chulae* collected in Okinawa and Iriomote Is., respectively.

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                 1                                                                         75
E. oxycephala    AGAGGCTCAAGTTGATAATCTCCGGCGTAAAGAGTGGTTAAGAAAAC-TAACACTAAAGCCGAACATCTTCAAGG
T. obscurus      ......C........C..ATG..........AT......T.T.C.GTA.TG.........TA...T.......A.
M. abei          ..G...C........C.GA.......A....A.....C...T.C...ACCC........T.A....C......AA
M. sp. 1         ..G...C........C.GA.......A....A.....C...T.C.G.ACCCG.......T.A....C......A.
M. chulae (#1)   ......C........C..AT.T....A....A....A....T.T.TGT.TTT.......T.A...........A.
M. chulae (#2)   ......C........C..AT.T....A....A....A....T.T.TGT.TTT.......T.A...........A.
M. parvus        ......C........C..G.C.....A....A.........T.C.GTA.T.........TTA...........A.
M. sp. 2         ......C........C..A.CT....A....A.........T.C.GTA.TT........T.A....C......A.

                 76                                                                        150
E. oxycephala    CTGTCATACGCACCCGAAGACATGAAGAACCCCTACGAAAGTAGCTTTAAAACTTCTGAACCCACGAAAGCCAGG
T. obscurus      .............T......A.A..G..C.TT.CG.......G........TAA.A.T.............T...
M. abei          ....T......T.T.........G....CC..T.C.........GA......GTAC.A.T..T..........T...
M. sp. 1         ....T......T.T.........G....CC.TT.C.........GA......GTAC.A.T..T..........T...
M. chulae (#1)   ........T..T...........G..GACC.TT.A.........GA.......CAC.A.T.C.T.........T...
M. chulae (#2)   ........T..T...........G..GACC.TT.A.........GA.......CAC.A.T.C.T.........T...
M. parvus        .A..T......C..T..G....G.....C.T...........GA.......TAA.A.A.C...........T...
M. sp. 2         .A.........T..T....G..G....GC.TT.C.........GA.......CAA.A.A.C...........T...

                 151                                                                       225
E. oxycephala    GCACAAACTGGGATTAGATACCCCACTATGCCTGGCCCTAAACACAAGCAGCTACCTCACACCTGCTGCTCGCCC
T. obscurus      AA............................A..............TGA....T.-.....CCTC...T...A
M. abei          AA............................A...T............ATA.GAC-...T..-T....T...A
M. sp. 1         AA...................T........A...T............ATA.AA.-...T..-T....T...A
M. chulae (#1)   A.................................T............ATA.AG.-...T..-T....T...A
M. chulae (#2)   A.................................T............ATA.AA.-...T..-T....T...A
M. parvus        AA............................A...T..........TGATA..A.-...T.A-T....T...A
M. sp. 2         AG............................A...T..........T..TAGTA.-...T.A-T....T...A

                 226                                                                       300
E. oxycephala    GGGAACTACGAGCAAAAGCTTAAAACCCAAAGGACTTGGCGGTGCTTTAGATCCACCTAGAGGAGCCTGTTCTAG
T. obscurus      ............CCC................................C......................
M. abei          ...G........CCC................................C......................
M. sp. 1         ...G........CCC................................C......................
M. chulae (#1)   ...G........CCC................................C.....................A
M. chulae (#2)   ...G........CCC................................C.....................A
M. parvus        ...G........CCC................................C......................
M. sp. 2         ............CCC................................C......................

                 301                                                                       375
E. oxycephala    AACCGATAACCCCCGTTCAACCTCACCCTTCCTTGTCTTCCCCGCCTATATACCGCCGTCGTCAGCTTACCCTGT
T. obscurus      ................A...........C.......ACT.................................
M. abei          ................A.........T.CT.....T..T...............................A.
M. sp. 1         ................A.........T.CT.....T..................................A.
M. chulae (#1)   ................A.........T.CT.....TA.T................................A.
M. chulae (#2)   ................A.........T.CT.....TA.T................................A.
M. parvus        ................A...........CT.....T.AT................................A.
M. sp. 2         ................A...........CT.....T..T..............A.................CA.
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                376                                                                       450
E. oxycephala   GAAGGACACATAGTAAGCAAAATTGGCATAGCCCAAAACGTCAGGTCGAGGTGTAGCGCATGGAGGGGGAAGAAA
T. obscurus     ..G....T......G....T.............T......C.................T...AGA..........
M. abei         .....CTTA.....G....C......T.A.A.........C...................C.AG.A.........
M. sp. 1        .....CTTA.....G....C..C...T.A.A.........C...................C.AG.A.........
M. chulae (#1)  .....C.CA.....G....C........C...........C...................C.AGAA.........
M. chulae (#2)  .....C.CA.....G....C........C...........C...................C.AGAA.........
M. parvus       ..G..CTTA..........C......T.A.A.........C...................C.AGA...C......
M. sp. 2        ..G..TTTA..........C..........A.........C.................T.C.AGA..........

                451                                                                       525
E. oxycephala   TGGGCTACATTCACTGACACAGTACATACGAATGATGCATTGAAACCAAACATCTGAAGGAGGATTTAGCAGTAA
T. obscurus     ................CAC....GA.A...........T.......-..G...T.A...................
M. abei         ..................C....GA.C.....CA....C.......-.G....TCA...................
M. sp. 1        ..................C....GA.C...G.CA....C.......-.G....TCA...................
M. chulae (#1)  ................C.C....GA.C.....CA....CC......-.G....TCA...................
M. chulae (#2)  ................C.C....GA.C.....CA....CC......-.G....TCA...................
M. parvus       .................TC.....A.......CA....C.......-.G....TCA...................
M. sp. 2        ..................C.....A.C......A....C.......-.G....T.....................

                526                                                                       600
E. oxycephala   GAAGGAAAACAGAGCGTTCCCCTGAAACTGGCCCTGAAGCGCGCACACACCGCCCGTCACTCTCCCCAAACTTTA
T. obscurus     .G...G..T-....T.CC.TT.................................................A.AGT
M. abei         .G..AG...-....T.CC.T...................................................AA--
M. sp. 1        .G..AG...-....T.CC.T..................................................TAA--
M. chulae (#1)  .G...G...-....T.CC.T........C.........................................TAA--
M. chulae (#2)  .G...G...-....T.CC.T........C.........................................TAA--
M. parvus       .G.A...T.-....T.CCTT......T............................................CA--
M. sp. 2        .G.....T.-....T.CC.T....................................................A--

                601                                                                       675
E. oxycephala   AAACA-GCCCATAAATAAGACACTAAGATCAAAAAGGGGAGGCAAGTCGTAACATGGTAAGTGTACCGGAAGGTG
T. obscurus     .GG..AATTT-.......ACA.AATCCTAG.C...........................................
M. abei         ..G-.CA.A.........ACA..A..C.AA.T...........................................
M. sp. 1        ..C-.CA.A.........ATA..A..C.AA.C...........................................
M. chulae (#1)  .....A..A.........TTA..A..C.AA.T...........................................
M. chulae (#2)  .....A..A.........TTA..A..C.AA.T...........................................
M. parvus       ..-..AATTAG.......ATA.TA...TAA.T...........................................
M. sp. 2        ..-..AATTG........ACA..A..TTAA.T...........................................

                676             tRNA-val      710
E. oxycephala   CACTTGGAAAAATC[AGAGCGTAGCTAAACTAGATA]
T. obscurus     ............C.[..GA..........A...-..]
M. abei         ..............[...A..........AC..-..]
M. sp. 1        ..............[...A..........AC..-C.]
M. chulae (#1)  ..............[...A..........A-..-..]
M. chulae (#2)  ..............[...A..........A-..-..]
M. parvus       T.............[...A..........-C..-C.]
M. sp. 2        ..............[...A..........-C..-..]
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