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Genetic differentiation in the genus *Uroconger* in the Indo-Pacific region

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ABSTRACT: Only a few species of congrid eels of the genus *Uroconger* (Congridae) have been described worldwide, but between these species each of the adult and larval morphologies are very similar. *U. lepturus* is the only species currently recognized in the Indo-Pacific, however, previous morphological counts of the total myomeres of *Uroconger* leptocephali suggest that more than 1 species may occur in this region. In this study, 1222 sites of the mitochondrial 16S rRNA gene were determined from 54 *Uroconger* leptocephali and one adult *U. lepturus* collected from 2 different regions of the Indo-Pacific to examine their genetic differentiations. The neighbor-joining tree based on Kimura's 2-parameter distances showed 4 major groups among the specimens examined, including one with the *U. lepturus* adult. The average inter-group genetic distances were 0.0348 to 0.0648, almost 20 times greater than the intra-group distances (0.0028 to 0.0036), and the former were larger than the distances between species in some other eels, such as those of the genera *Conger* and *Anguilla*. No clear geographic differentiation was found, but a highly significant difference was found between the 2 closest sister Groups A and B (F_{ST} = 0.90062, $p < 0.05$). However, the key characters of both the total number of myomeres and the position of the last vertical blood vessel overlapped among the genetic groups. These findings suggest the possibility of the presence of cryptic lineages within the genus *Uroconger* in the Indo-Pacific region.

KEY WORDS: *Uroconger* · Genetic differentiation · Indo-Pacific

INTRODUCTION

The congrid eels of the genus *Uroconger* are demersal tropical and subtropical fishes that live mainly along the continental margins and reach sizes of about 50 cm in length (Smith 1989a). They usually occur on soft mud or sandy bottoms and feed mainly on small benthic crustaceans (Froese & Pauly 2006), but little is known about their ecology and life history. The genus *Uroconger* presently only includes 2 or 3 species worldwide, but it is difficult to morphologically distinguish either the adults or the leptocephali of these species (Smith 1989a,b).

A single species, *Uroconger syringinus*, is known from both the western and eastern Atlantic (Smith 1989a, Froese & Pauly 2006). Another single species, *Uroconger lepturus*, is widely distributed from the western Indian Ocean to the western Pacific, as far north as Japan (Castle 1968, Smith 1989a), and it appears to be an abundant species of marine eel in the western Indian Ocean (Amir et al. 2005). A reported third species, *Uroconger erythraeus*, is restricted to the Red Sea (Klausewitz 1994), but its morphological differences from *U. lepturus* are still not clear.

Smith (1989a) examined morphological variation in *Uroconger syringinus* and *Uroconger lepturus* from different regions and found slight differences that suggested some regional divergence in morphological characters. Examinations of *Uroconger* leptocephali from the Indo-Pacific showed a wide range in the total number of myomeres (TM, corresponding to the total number of vertebrae [TV] in adults), suggesting more than 1 species of *Uroconger* may occur in the region (Smith 1989a).

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The development of molecular genetic techniques has revealed that various marine species considered to have large geographic distributions are often complexes of morphologically similar, but genetically distinct, species (Knowlton 2000). These techniques have played an important role in recognizing cryptic or sibling species in a variety of marine taxa such as invertebrates, fishes, and mammals (Knowlton 1993, Garcia-Rodriguez et al. 1998, Lima et al. 2005, Bickford et al. 2007). This has also been found to be true for bonefishes (Elopomorpha, Albuliformes) in the Indo-Pacific (Colborn et al. 2001), which also have a leptocephalus larva and are closely related to eels (Elopomorpha, Anguilliformes). The finding of several cryptic species of bonefishes suggests that this type of population differentiation in widely distributed species could exist in some species of eels such as *Uroconger*.

In the present study, the mitochondrial DNA (mtDNA) 16S rRNA genes of *Uroconger* leptocephali, and one *U. lepturus* adult, collected from different locations in the Indo-Pacific, were partially sequenced to examine their genetic differentiations. The genetic differences and speciation mechanisms within the genus *Uroconger* in the Indo-Pacific are then discussed.

MATERIALS AND METHODS

Collection and measurement. As part of a larger study on many taxa of congrid leptocephali in the Indo-Pacific region, 54 *Uroconger* leptocephali collected in the East China Sea (ECS), eastern Indian Ocean (EIO) and western North Pacific (WNP) (Table 1, Fig. 1) were examined morphologically and genetically. These collections were made during the 3 research cruises of KT-00-16 (5 to 6 December 2000, 29 stations), BJ-03-2 (5 to 20 June 2003, 25 stations), and KH-04-2 (2 July 2004, only 1 station south of Japan was included; a single deep tow using 4000 m of wire not specifically targeting leptocephali). The details of the first 2 sampling surveys have been previously described (Miller et al. 2002, Aoyama et al. 2007).

Leptocephali were collected using a large pelagic trawl (Isaacs Kidd Midwater Trawl [IKMT]) with an 8.7 m^2 mouth opening and either 1.0 or 0.5 mm mesh that was fished in both oblique and step tows (generally tows were in the upper 300 m or shallower). After collection, the leptocephali were sorted fresh from the plankton, measured to the nearest 0.1 mm total length (TL), and the 2 key morphological characters of an *Uroconger* leptocephalus, the TM and position of the last vertical blood vessel (LVBV), were counted in all specimens. They were identified at the genus level based on Smith (1989b), and then preserved in 95% ethanol.

Table 1. *Uroconger*. The collection data and total length (TL) of the 54 leptocephali and single adult specimens used in this study. The adult was *U. lepturus*. Locations: ECS: East China Sea, EIO: eastern Indian Ocean, WNP: western North Pacific

Location	Cruise	Date	N	TL (mm)
Leptocephali				
ECS	KT-00-16	5–6 Dec 2000	8	46.5–99.9
EIO	BJ-03-2	5–20 Jun 2003	45	16.0–143.4
WNP	KH-04-2	2 Jul 2004	1	111.7
Total			54	16.0–43.4
Adult				
Kagoshima Bay	–	Aug 28, 1998	1	381

Based on previous research (Smith 1989b, Ma 2006), the leptocephali included in the present study were both morphologically and genetically identified as belonging to the genus *Uroconger*. The leptocephali of *Uroconger* have a distinct single row of pigment spots along the midline, and are easily distinguished from other congrid leptocephali that have similar pigmentation by their high numbers of TM (Smith 1989b). A genetic comparison of these leptocephali to 6 known genera and 8 unknown taxa of congrid leptocephali

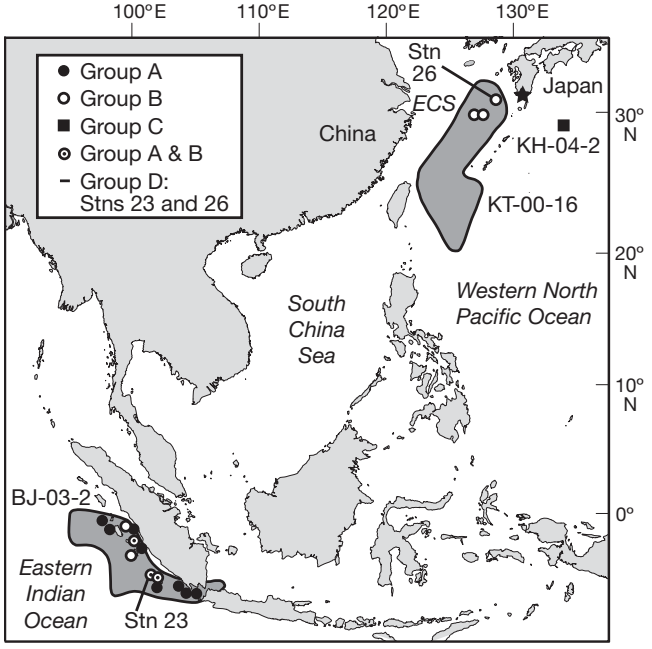

Fig. 1. *Uroconger*. Sampling locations of the *Uroconger* leptocephali (circles and squares) and the adult specimen of *U. lepturus* (black star) examined in this study. Stations where the leptocephali of each genetic group were collected are shown, along with the outline of the sampling regions of the KT-00-16 and BJ-03-2 surveys (dark grey shading). Stn 26 and Stn 23 are the locations where leptocephali genetically grouped with the adult specimen of *U. lepturus* were collected

clearly showed that the leptocephali morphologically identified as belonging to the genus *Uroconger* formed a distinct monophyletic clade (Ma 2006). In addition, there are no other known species of eels in the subfamily Congrinae present in the Indo-Pacific that have such a high range of TV (Froese & Pauly 2006, Ma 2006), except for *Gavialiceps* spp. whose leptocephali do not have lateral pigment (Karmovskaya 1994).

The adult specimen of *Uroconger lepturus* (TL = 381 mm) was collected during a trawling survey for benthic fishes in Kagoshima Bay carried out by Kagoshima University on 28 August 1998 (Table 1, Fig. 1). It was identified according to Hatooka (2000) and frozen after collection. This adult specimen was then deposited in the National Science Museum, Tokyo, Japan (NSMT-P71849).

PCR and sequencing. A portion of the body of the leptocephali and the musculature of the adult were used for DNA extraction and amplification. Total genomic DNA of each specimen was extracted by incubation in 500 µl of a 5% chelex solution (Bio Rad) at 98°C for 15 min. A fragment of the mtDNA 16S rRNA gene was amplified via polymerase chain reactions (PCRs) using the 4 oligonucleotide primers: L1854 (5′-AAA CCT CGT ACC TTT TGC AT-3′), L2510 (5′-CGC CTG TTT AAC AAA GAC AT-3′), H2582 (5′-ATT GCG CTA CCT TTG CAC GGT-3′), and H3058 (5′-TCC GGT CTG AAC TCA GAT CAC GTA-3′) (Inoue et al. 2000). PCRs were performed in a Model 9700 thermal cycler (Applied Biosystems) with a total of 15 µl reaction volume containing 0.2 mM of each deoxyribonucleotide triphosphate (dNTP), 1.5 µl of 10 × PCR buffer (Takara), 0.5 µM of each primer, 0.3 U of *Taq* DNA polymerase (Takara), and 0.6 µl of template. Typical amplification parameters were 30 cycles of denaturation at 94°C for 15 s, annealing at 50°C for 15 s, and extension at 72°C for 20 s after heating at 94°C for 5 min.

PCR products were electrophoresed on a 1.0% agarose gel L03 (Takara), and later stained with ethidium bromide for band characterization via ultraviolet transillumination. Double-stranded PCR products were purified using a Pre-Sequencing Kit (USB), that employs the shrimp alkaline phosphatase and exonuclease to remove excess dNTPs and oligonucleotides, and the purified products were subsequently used for direct cycle sequencing with dye-labeled terminators (Applied Biosystems). All sequencing reactions were performed according to the manufacturer's instructions with the same primers as those for PCR. Labeled fragments were analyzed on a Model 3100 or 3130 genetic analyzer (Applied Biosystems).

Sequence analysis. The DNA sequences were edited and analyzed with EditView v.1.0.1, AutoAssembler v.2.1 (Applied Biosystems), and DNASIS v.3.2 (HiTachi Software Engineering). Then all sequences were aligned using the alignment software package Clustal X 1.83 (Thompson et al. 1997) with default gap penalties, and subsequently adjusted by eye using MacClade v.4.05 (Maddison & Maddison 2002). These sequences were deposited in the DDBJ/ EMBL/GenBank nucleotide sequence databases with accession numbers: AB330934 to AB330988.

Analyses of genetic differentiation. The pairwise differences among samples were obtained by PAUP* v.4.0s (Swofford 2002), and Kimura's 2-parameter distances (Kimura 1980) were also calculated. Relationships between haplotypes were determined with Kimura's 2-parameter distance matrix using neighbor-joining by PAUP* 4.0s. The fixation indexes (F_{ST}) between locations or haplotype groups were calculated, and their significance was tested by the permutation test (10 000 permutations) using Arlequin 2.001 (Schneider et al. 2000).

RESULTS

The 54 *Uroconger* leptocephali examined had a wide range of TL (16.0 to 143.4 mm) (Table 1), but they were generally similar in morphology. Eight *Uroconger* leptocephali (46.5 to 99.9 mm TL) were collected in the northern area of the ECS and were present at 3 of the 29 stations that were sampled in the region (Fig. 1). Off West Sumatra in the EIO, 45 *Uroconger* leptocephali (16.0 to 143.4 mm TL) were collected at the stations closer to the coast (Fig. 1), and were present at 13 of the 25 stations that were sampled. A single 111.7 mm TL specimen was collected in the WNP just to the east of the ECS (Fig. 1). The TM and LVBV ranged widely from 206 to 240 and 56 to 69, respectively, in these specimens (Table 2). Both the TM and LVBV counts overlapped among the 3 locations, and higher ranges of both characters were seen in the EIO specimens (Fig. 2a,c).

Pairwise sequence comparisons showed that there were 0 to 79 (0 to 6.5%) site differences in the leptocephali examined based on 1222 sites of the mitochondrial 16S rRNA fragment. Among them, 1 specimen from the ECS (70.4 mm TL) and 1 specimen from the EIO (105.0 mm TL) showed very small sequence differences from the adult specimen of *Uroconger lepturus*, at only 6 and 8 sites (0.5, 0.7%), respectively.

The genetic analyses of the *Uroconger* sequence data showed that there were clear differences among the specimens. The neighbor-joining tree based on Kimura's 2-parameter distances identified 4 divergent groups, which were designated as Groups A, B, C and D (Fig. 3, Table 2). Most of the leptocephali were separated into 2 of the groups: Group A (N = 34, all were

collected in the EIO) and Group B (N = 17, collected from both the EIO and ECS). The 1 leptocephalus from the WNP was different from all the others and formed a separate Group C; and Group D consisted of the adult specimen of *U. lepturus* from Kagoshima Bay and a single leptocephalus from each of the ECS and EIO. The average intra- and inter-group genetic distances were 0.0028 to 0.0036 and 0.0348 to 0.0648, respectively (Table 3). The F_{ST} showed clear genetic differentiation between Groups A and B (F_{ST} = 0.90062, $p < 0.05$), and for Group A, a significant difference was observed between the 2 subgroups A1 and A2 (F_{ST} = 0.56085, $p < 0.05$). The genetic distance between these 2 subgroups was much lower, however (0.0051).

The TM and LVBV also overlapped in the 4 groups, and Group A had the widest range of TM (Fig. 2b,d, Table 2). Both the TM and LVBV of Group A (mean ± SD: 223 ± 7 and 65 ± 2, respectively) were a little higher than those of Group B (218 ± 7 and 63 ± 4), and they were significantly different for TM (*t*-test, p = 0.03), but not for LVBV (*U*-test, p = 0.17).

Table 2. *Uroconger*. The genetic groups, total length (TL), total number of myomeres (TM), and position of the last vertical blood vessel (LVBV) of the leptocephali examined in this study. ECS: East China Sea, EIO: eastern Indian Ocean, WNP; western North Pacific

Genetic group	Location	N	TL (mm)	TM Range	TM Mean ± SD	LVBV Range	LVBV Mean ± SD
A	EIO	34	16.0–143.4	208–240	223 ± 7	59–67	65 ± 2
B	EIO, ECS	17	20.8–126.0	208–227	218 ± 7	56–69	63 ± 4
C	WNP	1	111.7	220	–	66	–
D	ECS, EIO	2	70.4, 105.0	206, 213	210 ± 5	64, 68	66 ± 4

DISCUSSION

Genetic differentiation and cryptic lineages. The results of this study provided strong evidence of genetic differentiation in the genus *Uroconger* in the Indo-Pacific. Only one adult species of *Uroconger* is presently recognized across the wide areas of the Indo-Pacific, but this study identified at least 4 distinct genetic groups of *Uroconger* leptocephali, and most of them were sympatrically distributed in 2 locations (ECS and EIO) of the Indo-Pacific (Figs. 1 & 3). *Uroconger* leptocephali (Wouthuyzen et al. 2005) and adults (Froese & Pauly 2006) also have been found in the central Indonesian Seas, so the distributions of some of

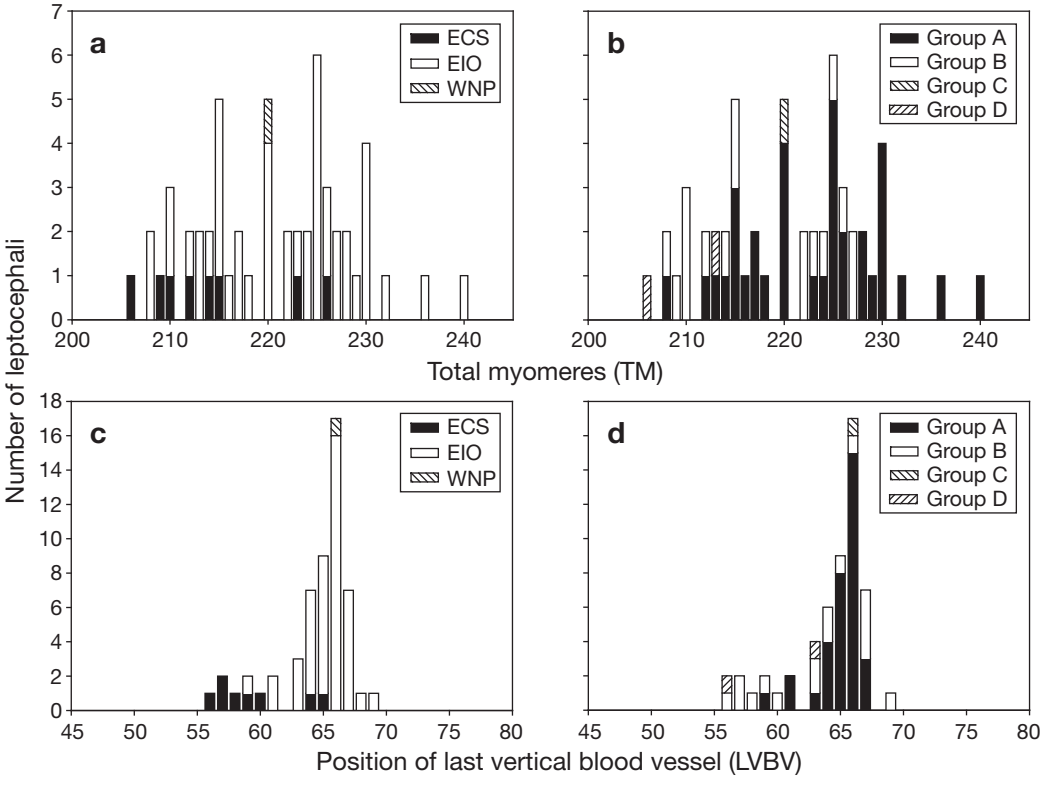

Fig. 2. *Uroconger*. Frequency distributions of (a,b) the total no. of myomeres, and (c,d) the position of the last vertical blood vessel of *Uroconger* leptocephali (a,c) collected at the 3 study area locations, and (b,d) of the 4 genetic groups that were mostly mixed among the study areas. ECS: East China Sea; EIO: eastern Indian Ocean; WNP: western North Pacific. N = 54

these groups may be continuous from the ECS to the EIO. Because the inter-group genetic distances (0.0348 to 0.0648) were almost 20 times greater than the intra-group distances (0.0028 to 0.0036), and the closest sister Groups A and B were genetically distinct based on the F_{ST} of 0.90062 ($p < 0.05$), these 4 groups may represent 4 distinct lineages. The genetic distances of the 16S rRNA gene between the 4 different groups in this study were much larger than those (0.0219) between the 2 species of east asian conger eels, *Conger japonicus* and *C. myriaster* (Ma 2006), and even larger than those (0.029 to 0.033) between the 2 species of Indonesian tropical freshwater eels, *Anguilla celebesensis* and *A. interioris* (Aoyama et al. 2000). The implication of the genetic differentiation within Group A (F_{ST} = 0.56085, $p < 0.05$) suggests there is some population structure within this group in the Indian Ocean, since the genetic distance between these subgroups was much lower (0.0051) than the inter-group genetic distances.

The *Uroconger* leptocephali examined here and in previous studies were very similar in morphology, but they have shown some geographic variation in TM. A clear variation in TM between the *Uroconger* leptocephali from the northeastern Indian Ocean (low range: 204 to 223) and those from Hawaii and New

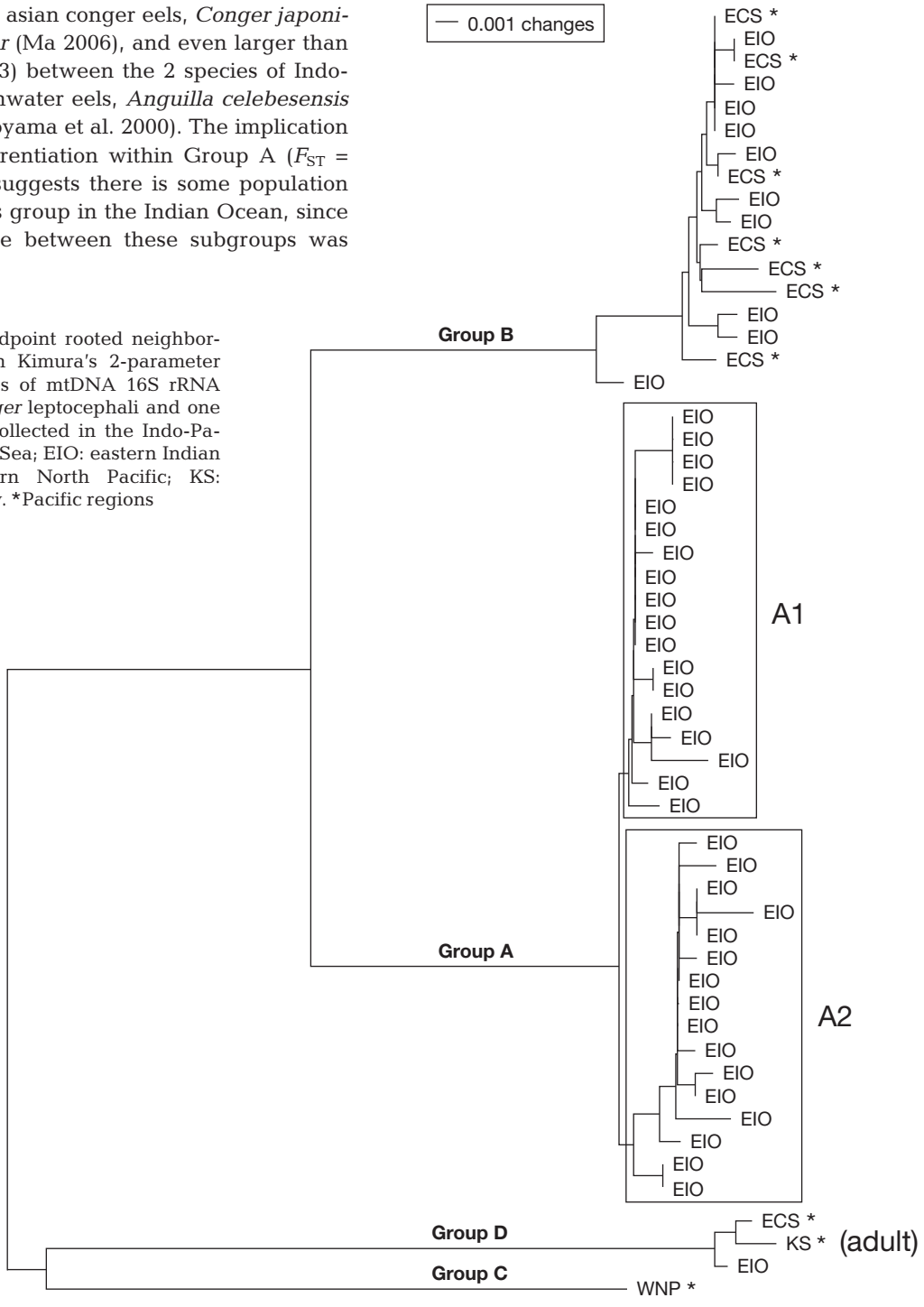

Fig. 3. *Uroconger*. Midpoint rooted neighbor-joining tree based on Kimura's 2-parameter distances of 1222 sites of mtDNA 16S rRNA gene from 54 *Uroconger* leptocephali and one adult of *U. lepturus* collected in the Indo-Pacific. ECS: East China Sea; EIO: eastern Indian Ocean; WNP: western North Pacific; KS: Kagoshima Bay. *Pacific regions

Table 3. *Uroconger*. Mean ± SD pairwise Kimura 2-parameter genetic distances within and between the 4 genetic groups examined in this study

Group	A	B	C	D
A	0.0036 ± 0.0020			
B	0.0348 ± 0.0020[a]	0.0034 ± 0.0020		
C	0.0557 ± 0.0017	0.0612 ± 0.0015	–	
D	0.0648 ± 0.0015	0.0598 ± 0.0015	0.0577 ± 0.0019	0.0028 ± 0.0005

[a]F_{ST} = 0.90062, p < 0.05

Caledonia (high range: 235 to 243) was found by Smith (1989a), but similar geographic differences were not observed in this study (Fig. 2). Although there were some differences in the mean values of TM and LVBV among groups, our *Uroconger* leptocephali were still difficult to distinguish using only morphology due to overlapped ranges in both characters (Table 2, Fig. 2). The adult morphology of *Uroconger* eels also may be very similar, because only one species has been recognized in the wide region of the Indo-Pacific, and even the differences between the Indo-Pacific (*U. lepturus*) and Atlantic (*U. syringinus*) species are very slight (Smith 1989a). This suggests the strong possibility of cryptic lineages existing in the genus *Uroconger* in the Indo-Pacific, which may be found to represent cryptic species after further research.

Using molecular genetic techniques, discoveries of cryptic species are common in a variety of marine organisms. Many of these are widely distributed species (Lessios et al. 1999, Knowlton 2000, Colborn et al. 2001, Quattro et al. 2006) such as *Uroconger lepturus*, or various types of coral reef species (Rocha et al. 2005, 2007). A general pattern in marine organisms that consist of cryptic species living in sympatry is that the species often have differences in ecology or life history, and after closer examination they are found to have subtle differences in morphology (Knowlton 1993, 2000, Rocha et al. 2005, 2007). This may also be applicable to the *Uroconger* eels in the Indo-Pacific.

Speciation mechanisms. It is difficult to determine the mechanisms of speciation of marine organisms because of the size and connectivity of marine areas, and the high potential for dispersal in a transglobal aquatic medium. Most speciation is thought to occur in allopatry, and the degree of occurrence of sympatric speciation is controversial (Barluenga et al. 2006). There are a few examples of apparent sympatric speciation in fishes (Johannesson 2001, Munday et al. 2004, Barluenga et al. 2006), but its occurrence is difficult to establish except in isolated habitats without potential barriers, such as freshwater lakes.

In the case of *Uroconger* eels, they have an extended pelagic larval period, because their leptocephalus larvae grow to sizes up to at least 143 mm (Smith 1989b, Wouthuyzen et al. 2005, this study), which, based on otolith studies on the leptocephali of other genera, must be greater than 100 d (Kimura et al. 2004, Ma et al. 2005). This long larval duration enables leptocephali to disperse over a geographically wide range, potentially increasing gene flow among different areas and reducing opportunities for allopatric speciation in areas connected by ocean currents. However, in the case of eels, dispersal capability itself does not necessarily translate into panmixia. Studies on the leptocephali of some congrid eels, such as *Conger myriaster* and *C. conger*, have suggested the possibility that these species may not be completely panmictic single spawning populations, despite their larval durations being very long (Kimura et al. 2004, Correia et al. 2006). Although the spawning areas of most congrid eels are not yet known, studies on anguillid eels have shown that they have evolved site-specific spawning areas to which they return for reproduction, regardless of having a widespread larval dispersion (Tsukamoto et al. 2002). If this type of spawning site fidelity is also present in the marine eel *Uroconger*, it could tend to facilitate genetic divergence based on the evolution of region-specific adult spawning and larval recruitment behaviors.

Therefore, the presence of different *Uroconger* sympatrically distributed in the various areas of the Indo-Pacific may have been caused by allopatric divergence. Over the entire Indo-Pacific, allopatric speciation could have occurred in various distant regions, including other areas such as Hawaii, the western South Pacific, or the western Indian Ocean, where specific spawning areas and recruitment mechanisms may have been required. Some species could have then expanded their ranges into areas occupied by other *Uroconger*, thus resulting in the sympatric distributions suggested in the present study. Allopatric divergence is also suggested as the predominant speciation mechanism in bonefishes, which also have a leptocephalus larva and sympatrically distributed cryptic species, and biogeographical boundaries such as the emergent shallow Sunda Shelf (located in the southern South China and Java seas) have apparently played an important role, influencing the genetic structure of bonefishes (Colborn et al. 2001). Similar biogeographic factors associated with multiple changes of sea level also may have contributed to the genetic differentiation in sympatrically distributed *Uroconger*.

Genetic analyses of *Uroconger* specimens from the Indonesian Seas and more distant regions such as the western Indian Ocean, Hawaii, and the South Pacific may provide additional clues about the history of genetic divergence throughout the range of this genus

and the possible presence of cryptic species. Future research should also examine the morphological, genetic, and life history differences in these eels using both adults and leptocephali to better understand the ecology and evolutionary history of the genus *Uroconger* in the Indo-Pacific region.

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