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Short Note

Evaluation of the population structure of *Anguilla bicolor bicolor* using total number of vertebrae and the mtDNA control region

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Abstract—The population structure of *Anguilla bicolor bicolor* was evaluated using their total number of vertebrae and genetic analyses of the mtDNA control region. Based on the likely geographic population structure of this subspecies in the Indian Ocean, the data were combined into East (Madras, Sumatra Island and Australia) and West groups (South Africa, Madagascar, Réunion and Seychelles) according to their sampling localities. Significant differences were not found in the range and mean total number of vertebrae between the East (N=74) and West groups (N=47) in the Indian Ocean, which were 107 to 113 (108.29±1.26), and 106 to 113 (109.60±1.47), respectively. Furthermore, the polymerase chain reaction—restriction fragment length polymorphism analysis for the mitochondrial control region using a total of 18 specimens from three localities showed no genetic differences between the East (N=14, Myanmar and Sumatra Island) and West groups (N=4, Madagascar) in the Indian Ocean. Morphological and genetic characters examined in the present study suggested no population structure for *A. bicolor bicolor* in the east and west side of the Indian Ocean, whereas they were ecologically assumed to be different populations. This contradiction suggested that these populations were in the beginning of speciation.

Key words: *Anguilla bicolor bicolor*, total number of vertebrae, mtDNA, control region, PCR-RFLP

Introduction

The shortfinned eel, *Anguilla bicolor*, has a relatively wide geographic distribution compared to most of the 18 species and subspecies of the genus *Anguilla*. Ege (1939) divided *A. bicolor* into the two subspecies *A. bicolor bicolor* and *A. bicolor pacifica* primarily because of slight differences in their means and ranges of total number of vertebrae. The distribution range of *A. bicolor bicolor* includes the coast of Africa, India, Srilanka, Bangladesh, Myanmar, northwestern Australia and Greater Sunda Islands, and the distribution range of *A. bicolor pacifica* includes the coast of China, Vietnam, the Philippines, Borneo Island, Sulawesi Island and New Guinea Island. A species such as this that has wide geographic distribution is generally composed of geographically separated populations (Avise 2000). Indeed, recent genetic studies revealed that the European eel, *A. anguilla*, possessed some apparent geographic genetic structure (Wirth and Bernatchez 2001), and *A. marmorata*, which is the most widely distributed freshwater eel has been clearly shown to have six geographic populations (Ishikawa 1998). However, the Japanese eel, *A. japonica*, and American eel, *A. rostrata*, were suggested to be single panmictic populations (Avise et al. 1986, Ishikawa et al. 2001, Wirth and Bernatchez 2003).

Jespersen (1942) reported that there were two different spawning areas of *A. bicolor bicolor* in the east and western side of the Indian Ocean. Recent studies on glass eels clearly showed remarkable differences in mean size and age at recruitment between both sides of the Indian Ocean (Budimawan 1997, Arai et al. 1999, 2001, Marui et al. 2001, Robinet et al. 2003). These facts suggest that *A. bicolor bicolor* on the east and west sides of the Indian Ocean are likely to have different ecological characteristics and to consist of different populations.

Our objectives were to compare the morphological and genetic differences between *A. bicolor bicolor* collected from the east and west sides of the Indian Ocean and to estimate the population structure of the species. In this study, we focused on the total number of vertebrae and the mitochondrial DNA (mtDNA) control region, which are representative taxonomic and genetic characters for the genus *Anguilla* that have been frequently analyzed to estimate the differences among or between the populations at a variety of levels.

Materials and Methods

Specimens

Prior to the study, we collected a total of 44 specimens (total length: 157–673 mm) from Madagascar (N=4), Réunion (N=5), Myanmar (N=5) and Sumatra Island (N=30) from May 1996 to June 2002. The specimens were preserved in 20% formalin for morphological observation after preserving a piece of liver tissue in 95% ethanol for genetic analysis. Specimens were identified morphologically as *A. bicolor bicolor* (data not shown) based on Ege (1939). In addition, we morphologically examined two specimens (No. 48830) of *A. bicolor bicolor* at the J. L. B. Smith Institute of Ichthyology, Grahamstown, South Africa (RUSI).

Total number of vertebrae

Except for nine specimens from Madagascar (N=4) and Myanmar (N=5) that were seriously damaged, a total of 37 specimens were X-ray photographed by Soft-X (Softex Co., Ltd.), and their total number of vertebrae (TV) was counted following Ege (1939). In addition, we added the total numbers of vertebrae for 84 specimens of *A. bicolor bicolor* described in Ege (1939) to the present data (Madagascar, Réunion and Seychelles (N=40), Madras (N=35) and Australia (N=9); Ege 1939).

Based on the likely geographic population structure of this species in the Indian Ocean, the data were grouped into East and West groups according to their sampling localities. These two groups were distinguished based on the wide geographic distance across the Indian Ocean separating the two locations where this subspecies is found (Fig. 1). Because our sample sizes were small, and Ege's (1939) sample mixed the three localities of Madagascar, Réunion and Seychelles on the west side of Indian Ocean, the data within the two groups were pooled for statistical analysis. The East Group contained Madras, Sumatra Island and Australia and the West group consisted of South Africa, Madagascar, Réunion and Seychelles. The TV data were tested with the Kolmogorov-Smirnov two-sample test using StatView (SAS Institute Inc., version 5.0), with $p<0.05$ as the criterion for significance.

PCR-RFLP of the control region

The PCR amplifications in most specimens were unsuccessful, probably due to improper preservation at the high temperatures of the tropical areas. Then, a total of 18 specimens were finally used for the polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) analysis. The number of specimens used for PCR-RFLP analysis was as follows: Madagascar (N=4), Myanmar (N=5) and Sumatra Island (N=9).

Total genomic DNA extraction was carried out following the standard protocol (Watanabe et al. in press). A fragment of $tRNA^{Thr}$ and $tRNA^{Pro}$ genes and a part of the control region was amplified by PCR with a pair oligonucleotide primers, L15774, 5′-ACATGAATTGGAGGAATACCAGT-3′ (Shields and Kocher 1991) and H16498, 5′-CCTGAAG-

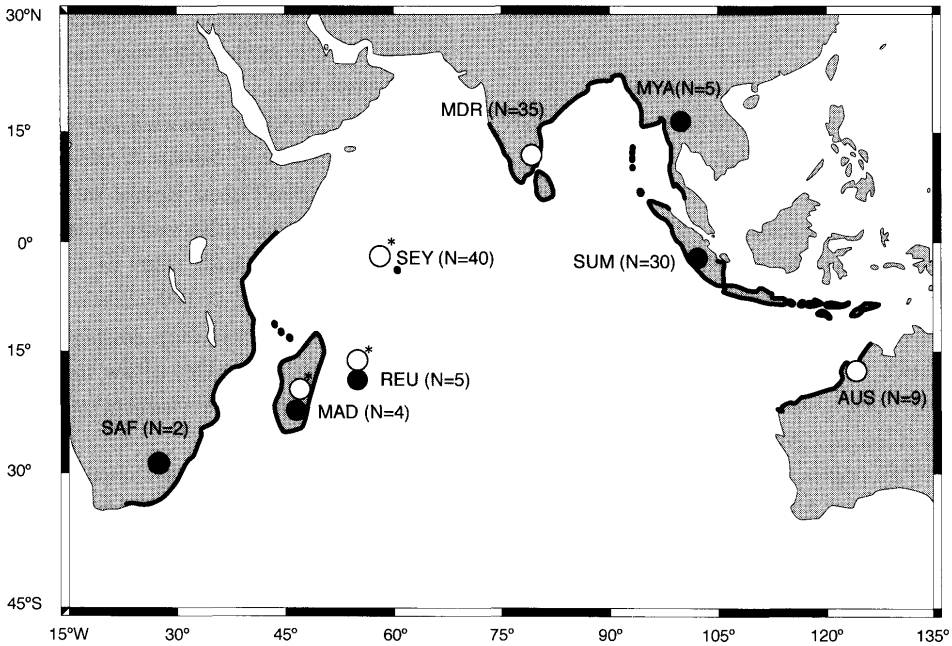

Fig. 1. The species range of *A. bicolor bicolor* (area covered by thick lines) based on Ege's (1939) study. The collection localities of specimens used in this and Ege's (1939) studies are shown by solid and open circles, respectively. The three capital letters are the abbreviaton of the localities as follows: SAF (South Africa), MAD (Madagascar), REU (Réunion), SEY (Seychelles), MDR (Madras), MYA (Myanmar), SUM (Sumatra Island), and AUS (Australia). * 40 specimens in parentheses of SEY indicates the total number of specimens from 3 localities (MAD, REU and SEY) in Ege's (1939) study.

TAGGAACCAGATG-3′ (Kocher et al. 1989). The PCR was carried out with the GeneAmp PCR system 2400 (Perkin-Elmer, Inc.), with a 25 μl reaction volume containing 13.8 μl of sterile distilled water, 2.5 μl 10×PCR buffer (Perkin-Elmer, Inc.), 2.5 μl dNTP (deoxynucleotide triphosphate) at 2 mM, 5 μl of each primer at 5 μM, 0.2 μl of *Taq* DNA polymerase (Perkin-Elmer, Inc.) and 1 μl of total DNA. Amplification parameters were 35 cycles of denaturation at 94°C for 30 s, annealing at 52°C for 30 s and extension at 72°C for 60 s. The PCR products were cleaved by the seven restriction enzymes, *Alu* I, *Eco* T22I, *Hha* I, *Hinc* II, *Mbo* I, *Msp* I and *Mva* I (Takara Shuzo Co., Ltd.). PCR-RFLP analysis and data analysis followed Watanabe et al. (in press). Restriction procedures were carried out in a 20 μl final volume containing 5 μl of PCR product, 10 units of restriction enzyme, and 2 μl of restriction enzyme buffer supplied by the manufacturers and incubated at 37°C overnight. RFLP was detected and compared with the positions of size markers (φX174-*Hinc* II digest, Takara Shuzo Co., Ltd.) using electrophoresis on 1% agarose gels with ethidium bromide staining. A 0-1 data matrix was constructed based on the absence (0) or presence (1) of the following 12 restricted fragment lengths (Watanabe et al. in press): 1 (>1057bp), 2 (=1057), 3 (>770, <1057), 4 (=770), 5 (>612, <770), 6 (=612), 7 (>495, <612), 8 (=495), 9 (>392, <495), 10 (=291, =392), 11 (>210, <291), 12 (=210). Hierarchical cluster analysis was carried out on this binary data set using SPSS (Professional Statistics, version 6.1) with the squared Euclidian distance measure, and UPGMA clustering (unweighted pair-group method using arithmetic averages).

Results and Discussion

The TV of the *A. bicolor bicolor* specimens examined in the present study ranged from 106 to 111 (mean±standard deviation, 108.60±1.82) in Réunion, 107 to 111 (109.57±1.10) in Sumatra Island, and 107 and 108 in the two specimens from South Africa. The combined data of *A. bicolor bicolor* in the present study and Ege's (1939) study had TV ranges in the East (N=74) and West groups (N=47) that were 107 to 113 (108.29±1.26), and 106 to 113 (109.60±1.47), respectively (Table 1). The Kolmogorov-Smirnov two-sample test for TV between the East and West groups showed no significant difference (p=0.94).

PCR-RFLP analysis for 18 specimens collected from three different localities showed a total of seven haplotypes. In the dendrogram estimated by the UPGMA analysis using a 0–1 data matrix, sampling localities were scattered without forming any geographic groups (Fig. 2). This indicated that the hypothetical East and West groups in *A. bicolor bicolor* were not found genetically, at least in the gene examined in the present study.

Table 1. Total and mean number of vertebrae of *Anguilla bicolor bicolor* in the West and East groups in the Indian Ocean. The East Group includes Madras, Sumatra Island and Australia and the West Group includes South Africa, Madagascar, Réunion and Seychelles.

TV	West group	East group
113	2	1
112	2	3
111	10	14
110	6	22
109	18	20
108	7	11
107	1	3
106	1	
N	47	74
mean	109.60	108.29
SD	1.47	1.26

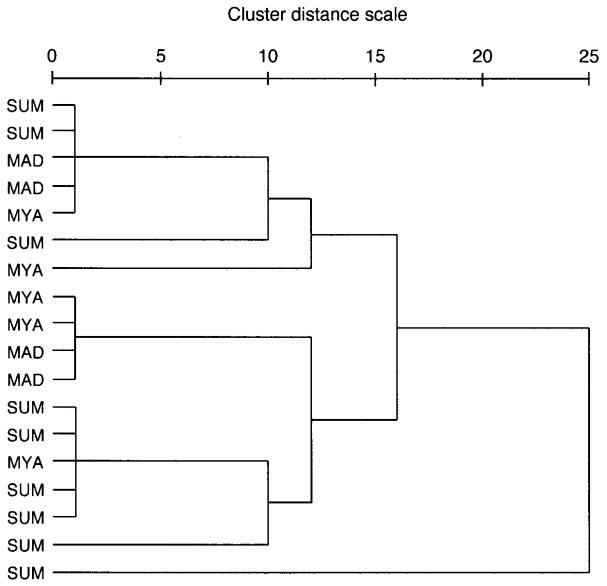

Fig. 2. Dendrograms obtained from the cluster analysis of the PCR-RFLP results for the control region of *A. bicolor bicolor*. Sample locations are in parentheses, MAD: Madagascar, MYA: Myanmar and SUM; Sumatra Island.

Morphological and genetic characters examined in the present study suggested that there is no population structure within *A. bicolor bicolor*, although the number of specimens was not enough to completely test the hypothetical population structure. The mitochondrial control region is one of the most rapidly evolving genes, and traditionally has been used for population genetic studies of animals (Bowen and Grant 1997, McMillan et al. 1999). However, recent advances in population genetics found more rapidly evolving genetic markers such as microsatellite DNA (Wirth and Bernatchez 2001), amplified fragment length polymorphism (AFLP) and single nucleotide polymorphisms (SNPs) (Bensch et al.

2002), which have revealed much lower levels of differentiated genetic structure of animals than has ever been found in the control region of mtDNA. It is still possible that *A. bicolor bicolor* has some population genetic structure consisting of only slight differences that were not detectable in the mitochondrial control region. Genetic analyses using much more specimens and rapidly evolving genetic markers will be required.

The most widely distributed freshwater eel species, the giant mottled eel, *A. marmorata*, has been found to have two genetically different populations, even in the mitochondrial control region, in the east and west sides of the Indian Ocean (Ishikawa 1998), although they were not different in morphological characters (Ege 1939). This suggested that *A. marmorata* inhabiting the east and west sides of the Indian Ocean have entirely different spawning areas and migratory mechanisms that prevents leptocephali from mixing with each other. Given that *A. bicolor bicolor* occurs on both sides of the Indian Ocean, it also could have a population structure similar to that of *A. marmorata*. Indeed, two different spawning areas have been suggested for *A. bicolor bicolor* in the Indian Ocean (Jespersen 1942), and the mean size and age at recruitment of this species at the east and west coasts drastically differed (Arai et al. 1999, Robinet et al. 2003). These facts suggested that *A. bicolor bicolor* is reasonably assumed to have different populations in the east and west sides of the Indian Ocean.

Recently, a conceptual model that superimposes both the life cycle of migratory fishes and the circular route of migration connecting the spawning area and growth habitat has been defined as a migration loop (Tsukamoto and Aoyama 1998, Tsukamoto et al. 2002). In principle, each species of diadromous fish has a migration loop peculiar to its life history and geographic distribution. This model is applicable to species or populations of the genus *Anguilla*. The deviation of a migration loop by some individuals of a species of the genus *Anguilla* would promote differentiation into different populations, and over time it is possible that they could become a new species. In this process the differentiation of ecological aspects such as geographic distribution, migratory behavior, and spawning areas occurs as the first step, preceding genetic or morphological differentiation, which is generally attributed to genetic isolation. In the case of *A. bicolor bicolor* in the Indian Ocean, it may be in the first step of differentiation because they showed genetic uniformity in the mitochondrial control region whereas considerable ecological differences occur between the east and west sides. In contrast, *A. marmorata* appear to be further differentiated with significant geographic genetic structure in the control region of mtDNA, although they do not differ morphologically. Furthermore, if we consider the Pacific and Indian Oceans, *A. bicolor pacifica* and *A. bicolor bicolor* showed considerable differences in both genetic (Watanabe 2001) and morphological characters (Ege 1939). This gradual diversification found in the genus *Anguilla*, from ecological traits to genetic and morphological differences, seem to reflect their speciation process from ecological groups to populations and eventually species.

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