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Identification of *Albula* sp. (Albulidae: Albuliformes) Leptocephalus Collected from the Southern Coastal Waters of Korea using Cytochrome *b* DNA Sequences

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Abstract – A single specimen of *Albula* leptocephalus (55.7 mm SL) was collected from the southern coastal waters of Korea using an aquatic lamp. It is characterized by having a ribbon-like body with a small head and a well-forked caudal fin. Although the general appearance was similar to the leptocephalus of *A. vulpes* including myomere counts and fin ray counts, the melanophore deposition was different from that of *A. vulpes*. This leptocephalus specimen was confirmed with *A. forsteri* using the cytochrome *b* mtDNA (*Cytb*) analysis. The genetic distance of *Cytb* between the present leptocephalus and *A. forsteri* is 0.006-0.038, which falls into the cutoff point separating *Albula* species into eight deep lineages including the four valid species. Its genetic characteristic have more similarities to those of Fiji than those of Hawaii and the Northern territory of Australia.

Key words – leptocephalus, *Albula forsteri*, cytochrome *b*, mitochondrial DNA, Jeju Island

1. Introduction

According to the recent taxonomical work of *Albula* (Albulidae) by Randall and Bauchot (1999), four valid species comprising *A. forsteri* Valenciennes in Cuvier and Valenciennes, 1847, *A. glossodonta* (Forsskål 1775), *A. nemoptera* (Fowler 1911), and *A. vulpes* (Linnaeus 1758) have been recognized worldwide. Genetically, these bonefishes are composed of eight deep lineages including the former four valid species (Colborn *et al*. 2001; Pfeiler *et al*. 2006). Of them, the three lineages including the anterior two valid species have been reported from the Indo-Pacific Ocean (Randall and Bauchot 1999; Colborn *et al*. 2001; Hidaka *et al*. 2004). From Korea, only one species, *A. forsteri* in the leptocephalus stage, has been recognized from the coastal waters of Geoje Island in the South Sea (Mori 1952 as *A. vulpes*; Chyung 1954, 1977 as *A. vulpes*; Kim *et al*. 2005 as *A. neoguinaica* Valenciennes).

The bonefishes are well known to have leptocephalus stage in their early life history as anguilliform fishes (Mochioka and Kozima 1988; Inoue *et al*. 2004; Miller and Tsukamoto 2004). The identification of leptocephalus to the species level is very difficult because the bonefishes have few common morphological characteristics between the leptocephauls and the adult stage. Recently, the molecular characteristics have been identified as useful tools for identification of fish eggs and larvae due to their constancy through the whole life (Aoyama *et al.* 1999; Akimoto *et al.* 2002; Shao *et al*. 2002; Rodríguez-Graña *et al.* 2004). In addition, the bonefishes have been well worked for cytochrome *b* (*Cytb*) mtDNA molecular characteristics by Colborn *et al*. (2001) and Pfeiler *et al*. (2006). To clarify the entity of *Albula* leptocephalus specimens collected from the southern coastal waters of Korea and fixed with the formalin, we tried to identify the present leptocephalus into the species level using both morphological method and molecular method using *Cytb* mtDNA sequence.

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2. Materials and Methods

A single leptocehalus specimen (NIBR-P0000002382, 59.2 mm in standard length, SL) was collected from the northern coast of Jeju Island, Korea using an aquatic lamp (200 W) and a hand net in October 27, 2005. Water temperature was 21°C on the surface. It was fixed in 5% formalin solution made with seawater in the field, and transferred into 70% ethanol within a few days after fixation. Measurements were made with the needlepoint calipers under a stereomicroscope to the nearest 0.1 mm. Observations of pigments and myomere counts were also conducted under the stereomicroscope. The present specimen is deposited in the fish collection of the National Institute of Biological Resources (NIBR), Korea.

Determination of Cytochrome *b* mtDNA sequences

Total genomic DNA was extracted from approximately 1 mg of the muscle tissue and the right eye using MN NucleoSpin-Tissue Kit (Macherey-Nagel, Germany) following the manufacturer's protocol. For PCR the preliminary trials with Cytochrome *b* (*Cytb*) primers H14803 (5'-TGCTAGGGTTGTGTTTAATT-3') and L15526 (5'-GTCTCCAAGAAGGTTAGGCGA-3') (Pfeiler *et al*., 2006) did not yield any PCR products, so that the new primer sets were designed specifically to amplify *Albula* species' DNA. Total three primer pairs were used yielding 170 bp, 173 bp and 220 bp of the sequence information from the *Cytb* genes: H14803, ALU-L-1 (5'-GTGCAACATGRATATARATACA-3'), ALU-H-2 (5'-TTGTATYTATATYCATGTTGC-3'), ALU-L-2 (5'-AAGAGATTTGTAATGACAGRA-3'), ALU-H-3 (5'-TACTGTCATTACAAATCTCTT-3'), ALU-L-3 (5'-AAATAAGGGTGAAACGGAACCTT-3').

PCR was conducted in 50 μl solution containing 1X PCR Buffer, 2.5 mM dNTP, 0.2 μM of each primer, five units of Super Taq polymerase (SUPER BIO, Korea), and 0.5-1.0 μg of genomic DNA. After preheating at 94 °C for five minutes, PCR reaction conditions consisted of 30 cycles of denaturation at 94 °C for one minute, annealing at 50 °C for one minute, and extension at 72 °C for two minutes; final extension at 72 °C for ten minutes. Double-stranded PCR products were ligated with the pCR2.1-TOPO vector using TOPO TA cloning kit (Invitrogen, California). The recombinant plasmids were then transformed into *E. coli* cells, which were cultivated on the LB plates with X-gal and ampicillin for the duration about 18 h. All sequencing reactions of the cloned DNA were conducted according to the manufacturer's protocol (Applied Biosystems Inc., Foster, California) using a 3730xl sequencer (Applied Biosystems, Inc.). The DNA sequences were analyzed using the computer program DNAssist V2.2 (Digital River GmbH, Germany). For diagnosis of its identity, the *Cytb* mtDNA sequence (GenBank accession numbers, EU555519) was compared with those of the *Albula* species deposited in EMBL/GenBank/DDBJ databases (Colborn *et al*. 2001; Pfeiler *et al*. 2006).

Construction of the haplotype phylogenetic tree

Phylogenetic trees were constructed by using the Neighbor-Joining distance method and the parsimony method using PHYLIP package (version 3.57c; Felsenstein 1995). Genetic distances were calculated using a Kimura-2-parameter (K2P) model (Kimura 1980) for the nucleotide substitution model in the Neighbor-Joining method (Saitou and Nei 1987). The bootstrap value in the parsimony tree was estimated by heuristic search with 1,000 replications.

3. Results and Discussion

Discription of Morphometric characteristics (Fig. 1; Table 1)

Dorsal fin rays 18; anal fin rays 8; pectoral fin rays ca. 12, incompletely developed; pelvic fin rays 9. Total myomeres 69; predorsal myomeres 54; preanal myomeres 68; prepelvic myomeres 36. Measurements in millimeters: standard length (SL) 55.7; predorsal length 44.0; prepelvic length 30.3; preanal length 53.0; head length 5.1; eye diameter 1.2; snout length 1.6. Proportion as % SL: head length 8.3; eye diameter 2.1; snout length 2.8; predorsal length 75.7; prepelvic length 52.2; preanal length 91.2.

Body elongated, highly compressed, and translucent. Head small and somewhat depressed. Upper jaw slightly longer than lower jaw. Sharp teeth on both jaws. Gut simple and straight, anus much near to posterior of body. Base of dorsal fin over 54th to 59th myomere. A dorsal finfold remained between posterior margin of dorsal fin and base of caudal fin. Base of anal fin below 68th to 69th myomere. A ventral finfold remained before anus. Pectoral fin membranous fan-like. Origin of pelvic fin below 36th to 37th myomere. Caudal fin well forked.

Melanophore deposition: two small melanophores on anteroventral region of upper jaw (lacking on right); two small melanophores on center of opercle (only one on right

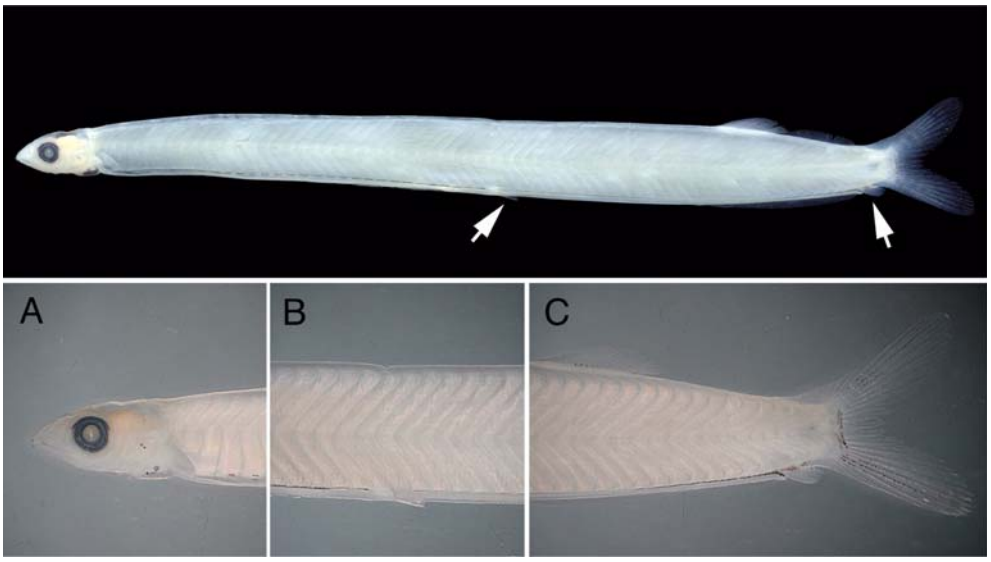

Fig. 1. Leptocephalus of *Albula* sp., NIBR-P0000002382, 55.7 mm SL, collected from northern coast of Jeju Island, Korea. Magnification of anterior (A), middle (B), and posterior (C) portions showing the melanophore deposition. Arrows indicate pelvic (anterior arrow) and anal (posterior) fins.

Table 1. Comparison of myomere counts and melanophore deposition between *Albula* sp. and *A. vulpes*

	Present specimen (*Albula* sp.)	*Albula vulpes*	
		Uchida *et al*. (1958)	Mochioka and Kozima (1988)
Total length (mm)	59.2 (n=1)	64.0 (n=1)	45.0-64.0 (n=4)
Total myomere	69	68	68-76
Predorsal myomere	54	51	51-61
Preanal myomere	68	-	65-76
Prepelvic myomere	36	32	32-38
Melanophore deposition			
on upper jaw	Present or absent	Absent	Absent
on operculum	Present	Absent	Absent
on anteroventral region of pectoral fin	Present	Absent	Absent
on dorsal, pelvic, anal fin bases	Present	Absent	Absent
on ventral midline of gut	Present	Present	Present
on caudal fin base	Present	Present	Present

side); two stellate melanophores on anteroventral region of pectoral fin; a series of melanophores on dorsal midline of gut from sixth myomere to origin of anal fin; eight melanophores on base of caudal fin; a series of melanophore on upper and lower fin rays of both caudal lobes; five branched melanophores on around center of dorsal fin base; several melanophores on basal region of pelvic and anal fins.

Molecular characteristics

A total of 546 bp of *Cytb* mtDNA sequences (haplotype ALBK1) was determined from the present single *Albula* leptocephalus specimen using the three sets of primers. The partial *Cytb* mtDNA sequence was aligned from the three partial sequences comparing to those of *Albula* species (Colborn *et al*. 2001; Pfeiler *et al*. 2006). Its sequence was very similar to those of *A. forsteri* among the eight deep lineages of *Alubula* species (Colborn *et al*. 2001; Pfeiler *et al*. 2006). The present leptocehalus haplotype (ALBK1) was different from the haplotypes of *A. forsteri* (ALB45, ALB47 and ALB54; Colborn *et al*. 2001) by only amino acids at positions 18, 95, and 177, respectively (Table 2). The K2P genetic distance was 0.006-0.038 between ALBK1 and *A. forsteri* (Fig. 2).

Table 2. The similarity of *Cytb* nucleotide sequence of NIBR-P0000002382 compared with other *Ablula forsteri* and variable amino acid position in consensus sequences of the *Cytb* gene segment of *Albula* spp. Translated from *Cytb* nucleotide sequences in Pfeiler *et al*. (2006) and the present study, unless indicated otherwise. Amino acid abbreviation: F, phenylalanine; I, isoleucine; M, methionine; T, threonine; V, valine. Dots indicate identical amino acids relative *Albula forsteri* (AF11765)

Species	Accession No.	Geographic Area	Identity (%)	Position		
				118	195	177
A. forsteri	AF11765	Indo-West Pacific (Fiji)	99	T	M	F
A. forsteri	AF11763	Central pacific (Hawaii)	96	I	T	V
A. forsteri	AF11764	Central pacific (Hawaii)	96	•	•	V
Present specimen	EU555519	South Korea (Jeju)	-	•	•	•

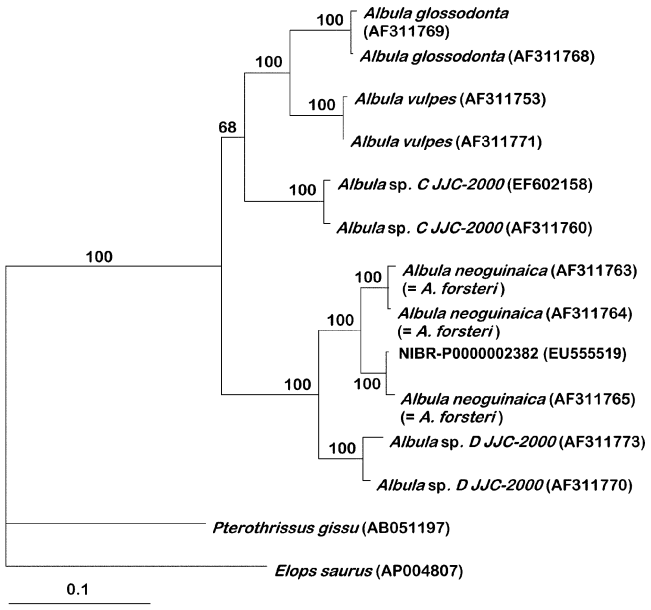

Fig. 2. Phylogenetic tree based on partial *Cytb* nucleotide sequences showing the relationship between NIBR-P0000002382 and other members of the genus *Albula*. The tree is based on the K2P distance model and the neighbour-joining method. Bootstrap values > 60% of 1,000 replications are shown. Bar indicates genetic distance of 0.1.

Identification

The present leptocephalus specimen collected from Jeju Island, Korea was readily identified as a member of either Albulidae or Pterothrissidae (Albuliformes) due to having a well-developed forked caudal fin and a small dorsal fin positioned far forward of anal and caudal fins (Smith 1979; Richards, 1984; Mochioka and Kozima 1988; Miller and Tsukamoto 2004). These two albuliform families contain monotypic genus *Albula* and *Pterothrissus*, respectively (Nelson 2006). According to Smith (1979), both *Albula* (Albulidae) and *Pterothrissus* (Pterothrissidae) leptocephali have the origin of anal fin well behind the dorsal. However, they also could be separable by the distance between the posterior edge of the dorsal fin and origin of anal fin and by the shape of snout (Richards 1984; Mochioka and Kozima 1988; Miller and Tsukamoto 2004), *i.e*., the distance in the former is about 2.5 times the dorsal fin base and the shape of snout is short round, whereas in the latter its distance is about 6-7 times and the shape of snout is prolonged. The distance between posterior edge of the dorsal fin and origin of anal fin in the present leptocephalus is 2.3 times the dorsal fin base, and the shape of snout is short round (Fig. 1); therefore, it was identified as a species of *Albula*.

In the myomere counts, the present *Albula* leptocephalus has somewhat more numbers than those of *A. vulpes* from Geoje Island, whereas its numbers are overlapped by those of *A. vulpes* from Japanese specimens (Table 1). It is noted that the melanophore deposition in *A. vulpes* by Uchida *et al*. (1958) and by Mochioka and Kozima (1988) is limited on the abdomen and caudal fin, however, the present *Albula* leptocephalus also has on the anterior portion of upper jaw, operculum, anteroventral region of the pectoral fin, and base of the dorsal, pelvic, and anal fins (Fig. 1; Table 1). There is difficulty in that we identify the leptocephalus from Geoje Island and also the present *Albula* leptocephalus specimens into one of *A. vulpes*, *A. forsteri*, *A. glossodonta* or of other species. The reason is that morphological characteristics between leptocephalus and adult are few common and also there are no reports based on actual specimens of adults or juveniles from Korea to date. After all, we failed to identify the present *Albula* leptocephalus into the species level based on the morphological method only.

On the contrary, to identify the fish eggs and larvae into species level, the molecular tools have more merits than the traditional morphological identification methods when the morphological identification keys are insufficient or very similar between species (Aoyama *et al.* 1999; Akimoto *et*

al. 2002; Shao *et al*. 2002; Rodríguez-Graña *et al.* 2004). The molecular tools have recently been used for the identification of species and populations as well as the analysis of phylogenetic relationship among species (Okazaki *et al*. 1996; Avise 2000; Hebert *et al*. 2003; Blaxter 2004). Therefore, we examined the *Cytb* mtDNA sequences of the present *Albula* leptocephalus species because the information about *Cytb* mtDNA sequences of eight deep lineages including the four valid *Albula* species is well known (Colborn *et al*. 2001; Pfeiler *et al*. 2006).

The K2P genetic distance among *Albula* species is 5.56-30.60% (Colborn *et al*. 2001). Here, the 5.56% of the genetic distance had been used as the cutoff point in the eight deep lineages of the *Albula* species. This cutoff point falls within the range of values reported for sister species based on the *Cytb* mtDNA (Johns and Avise 1998). We obtained the 546 bp of the *Cytb* mtDNA sequences composed of three sets of partial sequences from a single leptocephalus specimen (haplotype ALBK1). Its sequences are similar to that of *A. forsteri* among the eight deep lineages (Colborn *et al*. 2001; Pfeiler *et al*. 2006). The genetic distance between ALBK1 and *A. forsteri* was 2.9-4.4%, which was lower than the cutoff point in the eight deep lineages of the *Albula* species. It suggests that the haplotype ALBK1 seem to be conspecific with *A. forsteri*. In addition, the haplotype ALBK1 has higher similarity in Fiji haplotypes than in Hawaii and Northern Territory haplotypes (Table 2; Fig. 2).

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