African Journal of Biotechnology Vol. 11(25), pp. 6641-66446, 27 March, 2012
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB10.2521
ISSN 1684–5315 © 2012 Academic Journals

Full Length Research Paper

Taxonomic status of the black porgy, *Acanthopagrus schlegelii* (Perciformes: Sparidae) inferred from mitochondrial genes

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Accepted 14 November, 2011

The black porgy *Acanthopagrus schlegelii* (Bleeker, 1854) is a commercially important fish distributed in estuarine and coastal waters of the west Pacific Ocean. Based on body color pattern, two subspecies *A. schlegelii schlegelii* (dark-unicolored specimens) and *A. schlegelii czerskii* (striped specimens) were suggested by some taxonomists. However, due to the morphology similarity, the phylogenetic relationship between the two subspecies has been long-lasting confused. The purpose of this study was to evaluate the taxonomic status of the two subspecies by two mitochondrial genes, cytochrome oxidase subunit I (COI) and control region (CR). Eight *A. schlegelii schlegelii* and eight *A. schlegelii czerskii* were collected from the coastal waters of the Yellow Sea, the East China Sea and the South China Sea. The genetic diversity, genetic distance and phylogenetic relationship of the two subspecies were analyzed and compared. The genetic diversity indices were close to each other in COI, but more different in CR. The mean genetic distances between the two subspecies was 0.0015 in COI and 0.0051 in CR, respectively. These values are much lower than those found for interspecific COI and CR comparisons among some species of *Acanthopagrus* (0.0667 to 0.0954 in COI and 0.2267 to 0.2480 in CR). Moreover, haplotypes of the two subspecies did not form reciprocal monophyletic clades in the phylogenetic trees based on the two mitochondrial genes. These results indicate that the genetic distance between the two subspecies, *A. schlegelii schlegelii* and *A. schlegelii czerskii*, was at the intraspecies level; they should be classified into the same species: *A. schlegelii*. It is suggested that *A. schlegelii schlegelii* and *A. schlegelii czerskii* should be regarded as the junior synonyms of *A. schlegelii*.

Key words: *Acanthopagrus schlegelii schlegelii*, *A. schlegelii czerskii*, mitochondrial DNA, molecular phylogeny, taxonomic status.

INTRODUCTION

The genus *Acanthopagrus* was first proposed by Peter (1855) as a subgenus of the genus *Chrysophrys* (Pagrus) with type species, *Ch. vagus* Peter 1852, Sparus berda Forsskål 1755. At present, the genus *Acanthopagrus* has become an independent genus, and comprises about 13 species (Kume and Yoshino, 2008). Fishes of the genus *Acanthopagrus* are important fishery species widely distributed in tropical and temperate waters in the Indo-West Pacific Ocean. Several of them, such as *A. berda* (Forsskål, 1775), *A. latus* (Houttuyn, 1782) and *A. schlegelii* (Bleeker, 1854) also have a high value in aquaculture. However, some species of the genus are in obscure taxonomic status because of their morphological similarity. The black

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Table 1. Sampling data of *A. schlegelii schlegelii* and *A. schlegelii czerskii* including collection location, sample size, standard length and haplotypes.

Sample	Collection location	Sample size	Standard length (mm)	COI haplotype	CR haplotype
A. schlegelii schlegelii	Qingdao	3	50 – 90	C2	D4
	Xiamen	3	214 – 263	C1	D1 – D2
	Zhanjiang	2	151 – 158	C1	D2 – D3
A. schlegelii czerskii	Qingdao	5	40 – 85	C1, C2	D4, D7 – D10
	Xiamen	3	212 – 221	C1 – C3	D4 – D6

porgy *A. schlegelii* is one of the most confused species of them.

A. schlegelii (=*Chrysophrys schlegelii*) was originally described by Bleeker (1854) based on two synotypes from Nagasaki, Japan, with unicolored dark ash-grey body and a silver-white abdomen. Berg (1914) reported another black porgy, *Sparus swinhonis czerskii* (=*A. schlegelii czerskii*) from the Tuman estuary, which had the appearance similar to *A. schlegelii*, but is characterized by six to seven transverse broad dark stripes. Accordingly, *A. schlegelii schlegelii* (dark-unicolored specimens) and *A. schlegelii czerskii* (striped specimens) have been recognized as the two different subspecies due to their different color patterns (Lindberg and Krasyukova, 1969; FAO Fishbase, 2010). However, Dolganov et al. (2008) suggested that the two subspecies should be invalid subspecies because both of them were recorded to co-occur at the same distribution areas in the Sea of Japan, the Yellow Sea and the East China Sea. Recently, Kharin and Markevich (2010) regarded that *S. swinhonis czerskii* (=*A. schlegelii czerskii*) is a junior subjective synonym of *A. schlegelii* after examining the morphological characters of the synotypes of the two species. Up to now, no molecular methods are used to elucidate the genetic differences between *A. schlegelii schlegelii* and *A. schlegelii czerskii*.

With the rapid advance of molecular techniques in recent years, mitochondrial DNA (mtDNA) gene has been widely applied to the studies of taxonomy and phylogenetic evolution in fishes (Avise, 2000; Saitoh et al., 2006). Within the mitochondrion genome, cytochrome oxidase subunit I (COI) barcoding is regarded as one of the efficient tool to both fish species classification and phylogeny (Ward, et al., 2005). The control region (CR) is known to have a dual nature of both high variability and conservatism, which is very useful for population genetics analysis and interspecific comparison (especially for the closely related species) (Chen et al., 1998; Liu, et al., 2007). In this study, we employed the sequences of COI and CR to evaluate taxonomic status of the black porgy, *A. schlegelii schlegelii* (dark-unicolored specimens) and *A. schlegelii czerskii* (striped specimens). The results of this study will provide important molecular genetic evidences for the identification and delineation of *A. schlegelii*.

MATERIALS AND METHODS

Sample collection

A total of 16 individuals of the black porgy were sampled from coastal waters of Qingdao (Yellow Sea), Xiamen (East China Sea) and Zhanjiang (South China Sea) from 2009 to 2010 (Table 1). The voucher specimens were deposited in the Ocean University of Guangdong. Muscle samples were preserved in 95% ethanol for DNA extraction.

For mtDNA identification, the samples were divided into two groups according to their color patterns. Eight dark-unicolor specimens sampled from the coastal waters of Qingdao, Xiamen and Zhanjiang were assigned to *A. schlegelii schlegelii*, with a range of standard length of 50 to 263 mm, including juvenile (Qingdao specimens) and mature (Xiamen and Zhanjiang specimens) individuals (Table 1). The other eight striped specimens sampled from the coastal waters of Qingdao and Xiamen were assigned to *A. schlegelii czerskii*, with a range of standard length of 40 to 221 mm, also including juvenile (Qingdao specimens) and mature (Xiamen specimens) individuals (Table 1).

PCR amplifying and sequencing

Genomic DNA was isolated from muscle tissue by a standard phenol-chloroform method (Sambrook et al., 1989). The COI was polymerase chain reaction (PCR) amplified with the primers F1 (5'-TCAACCAACCACAAAGACATTGGCAC-3') and R1 (5'-TAGACTTCTGGGTGGCCAAAGAATCA-3') (Ward et al., 2005). The 5'-end of CR was amplified with the primers L (5'-TTAGTATGGTGACAATGCAT-3') and H (5'-GACACCATTAACTTATGCAA-3') (Liu et al., 2004).

PCR was performed in a 50 µl reaction volume containing 20 to 50 ng of template DNA, 5 µl of 10×reaction buffer, 1.5 mM of $MgCl_2$, 200 µM of dNTP mixture, 0.2 µM of each primer, and 1.5 units of *Taq* DNA polymerase (Takara, Japan). The PCR amplifications were performed on a Biometra thermal cycler (Biorad, USA) under the following conditions: A denaturation step at 94°C for 3 min, followed by 35 cycles consisting of 94°C for 45 s, 52°C for 45 s, 72°C for 45 s and a final extension at 72°C for 7 min. All sets of PCR amplifications included a negative control reaction tube in which all reagents were included, except the template DNA. PCR products were purified with the Gel Extraction Mini Kit (Tiangen, Beijing). The purified products were sequenced in Shanghai Invitrogen Biotechnology Company using an ABI Prism 3730 (Applied Biosystems, USA) automatic sequencer with both forward and reserve primers.

Data analysis

In order to determine the genetic distance between *A. schlegelii*

Table 2. Comparative genetic diversity indices of *A. schlegelii schlegelii* and *A. schlegelii czerskii* based on COI and CR sequences.

Genetic diversity indice	COI		CR	
	A. schlegelii schlegelii	***A. schlegelii czerskii***	***A. schlegelii schlegelii***	***A. schlegelii czerskii***
Sequence length (bp)	651	651	453	453-454
Sample size	8	8	8	8
Number of variable sites	2	3	3	14
Number of haplotypes	2	3	4	7
Haplotype diversity (*h*)	0.5357±0.1232	0.6071±0.1640	0.8214±0.1007	0.9643±0.0772
Nucleotide diversity (*л*)	0.0017±0.0014	0.0017±0.0014	0.0027±0.0021	0.0091±0.0058
Mean number of pairwise differences (*k)*	1.0714±0.7856	1.1071±0.8042	1.2269±0.8663	4.1871±2.3246

schlegelii and *A. schlegelii czerskii*, we integrated sequences of the following congener species from GenBank: *A. australis* (COI: DQ107855; CR: AF381056), *A. berda* (COI: EF607297; CR: AM992246), and *A. latus* (COI: GU207344; CR: EF506764). The homologous sequences of *Rhabdosargus sarba* (COI: FJ238020) were used as outgroup.

Sequence chromatograms of both directions in each sample were viewed and edited using the software Dnastar (DNASTAR Inc., USA). Sequences were multiplied and aligned using Clustal X (Thompson et al., 1997). Haplotypes of were defined by DnaSP4.0 (Rozas et al., 2003) and submitted to GenBank directly (GenBank accession numbers: HQ846830-HQ846832 for COI and HQ846833-HQ846842 for CR). Genetic diversity indices such as number of haplotypes, number of polymorphic sites, haplotype diversity (*h*), nucleotide diversity (*л*), and the mean number of pairwise differences (*k*) were obtained by the program ARLEQUIN 3.1 (Excoffier et al., 2006). Base composition and genetic distance based on Kimura-2 parameter was calculated using MEGA 4.0 (Tamura et al., 2007). The phylogenetic relationships among haplotypes of the two subspecies and their closely related species were reconstructed using the neighbor-joining (NJ) method implemented in MEGA 4.0. The NJ trees were evaluated with 1000 bootstrap replicates.

RESULTS

Sequence variance and base composition

COI and CR were sequenced in 16 specimens, respectively. After multiple sequence alignment, the consensus sequences of COI and CR (excluding both tRNA-Thr and tRNA-Pro genes) were 651 bp and 453 to 454 bp, respectively. All analyses of this study were based on these consensus sequences. For COI, the average nucleotide composition of *A. schlegelii schlegelii* was the same as *A. schlegelii czerskii*. The content of A, T, C, G was 24.1, 31.4, 26.6 and 17.9%, respectively, showing an obvious anti-guanine bias phenomenon. Two variable sites were found and two haplotypes (C1, C2) were defined in the specimens of *A. schlegelii schlegelii*. Three variable sites were found and three haplotypes (C1–C3) were defined in the specimens of *A. schlegelii czerskii*. Both C1 and C2 were shared by the two subspecies (Table 1). The genetic diversity indices of *A. schlegelii schlegelii* (*h* = 0.5357, *л* = 0.0017, *k* = 1.0714) were close to those of *A. schlegelii czerskii* (*h* = 0.6071, *л* = 0.0017, *k* = 1.1071) (Table 2).

For CR, the average nucleotide composition of *A. schlegelii schlegelii* was also the same as *A. schlegelii czerskii*. The content of A, T, C, G was 38.0, 30.3, 20.7 and 11.0%, respectively, indicating that the region is A, T rich. Three variable sites were found and four haplotypes (D1–D4) were defined in the specimens of *A. schlegelii schlegelii*. Fourteen variable sites were found and six haplotypes (D4, D6–D10) were defined in the specimens of *A. schlegelii czerskii*. Only D4 was shared by the two subspecies (Table 1). The genetic diversity indices of *A. schlegelii schlegelii* (*h* = 0.8214, *л* = 0.0027, *k* = 1.2269) were lower than those of *A. schlegelii czerskii* (*h* = 0.9643, *л* = 0.0091, *k* = 4.1871) (Table 2).

Genetic distance

Based on Kimura-2 parameter, the mean genetic distance within *A. schlegelii schlegelii* specimens was 0.0017 (0 to 0.0031) for COI and 0.0018 (0 to 0.0044) for CR. The mean genetic distance within *A. schlegelii czerskii* specimens was 0.0017 (0 to 0.0046) for COI and 0.0091 (0 to 0.0157) for CR. The genetic distance between *A. schlegelii schlegelii* and *A. schlegelii czerskii* was 0.0015 for COI and 0.0051 for CR (Table 3). The genetic distances among other three species of *Acanthopagrus* were 0.0667 to 0.0954 in COI, and 0.2267 to 0.2480 in CR (Table 3), which were much higher than those between two subspecies for the two genes. The distances between the out-group *R. sarba* and *Acanthopagrus* species were greater than those for all pairwise comparisons between *Acanthopagrus* species for both genes.

Phylogenetic relationship

The results of phylogenic analysis showed that the haplotypes of *A. schlegelii schlegelii* and *A. schlegelii czerskii* did not form reciprocal monophyletic clades in the NJ trees based on either COI or CR; and the close relationship among the haplotypes of the two subspecies was supported by high confidence levels (100%) for both genes (Figures 1 and 2). In contrast, the relationship

Table 3. Pariwise genetic distances with the Kimura–2 parameter model among *Acanthopagrus* species and the outgroup *R. sarba* based on COI (below diagonal) and CR (above diagonal) sequences.

Species	1	2	3	4	5	6
A. schlegelii schlegelii		0.0051	0.2184	0.1981	0.1997	0.3661
A. schlegelii czerskii	0.0015		0.2184	0.1977	0.1997	0.3586
A. australis	0.0883	0.0885		0.2424	0.2480	0.4252
A. berda	0.0617	0.0615	0.0667		0.2267	0.3832
A. latus	0.0865	0.0867	0.0954	0.0880		0.3963
R. sarba	0.1585	0.1583	0.1445	0.1528	0.1569	

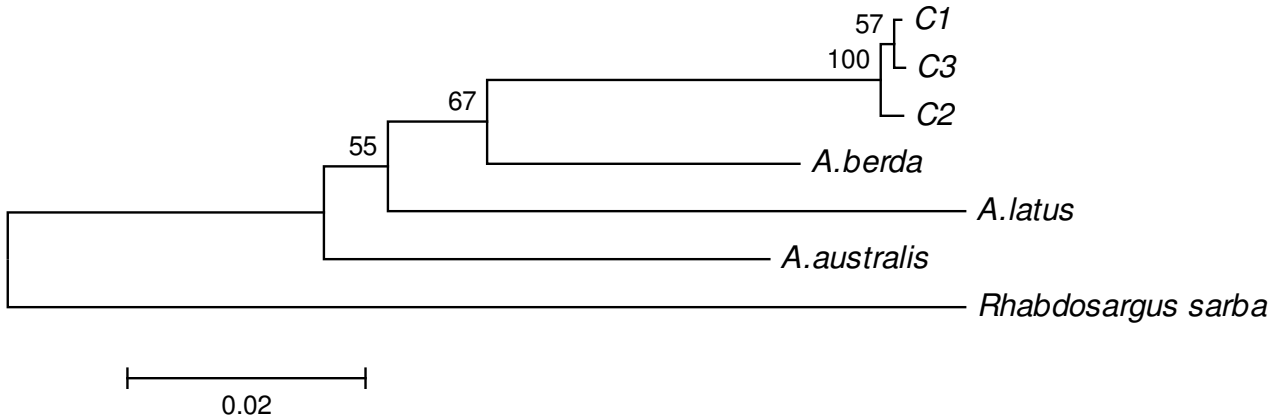

Figure 1. Neighbor-joining tree for the haplotypes of *A. schlegelii schlegelii* (C1–C2) and *A. schlegelii czerskii* (C1–C3) as well as their closed species based on COI sequences. Bootstrap support of >50% in 1000 replicates is shown above branches.

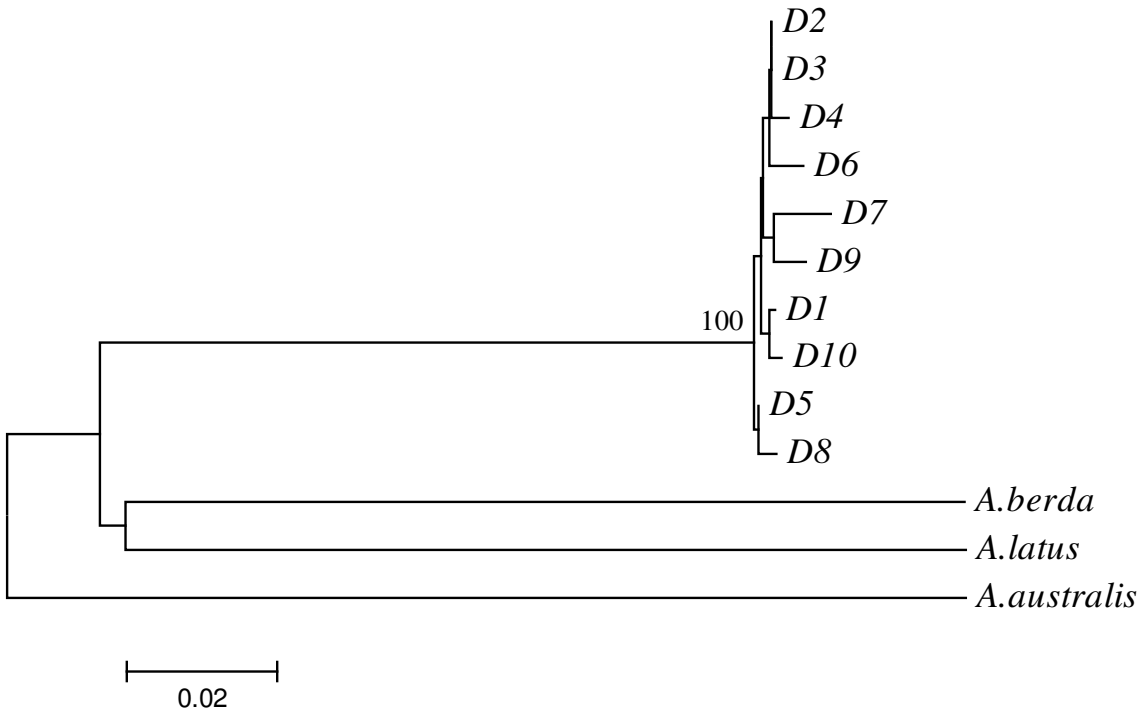

Figure 2. Neighbor-joining tree for the haplotypes of *A. schlegelii schlegelii* (D1–D4) and *A. schlegelii czerskii* (D4, D6–D10) as well as their closed species based on CR sequences. Bootstrap support of >50% in 1000 replicates is shown above branches.

between the two subspecies and other three species of *Acanthopagrus* are unresolved, because of the two sets of relationship for two mitochondrial genes and their relatively lower confidence (67% for COI and 42% for CR).

DISCUSSION

The number of hard and soft in rays, dentition and color pattern are the main morphological characteristics in discrimination of the Sparidae species (Smith and Smith, 1986). According to body color pattern, Lindberg and Krasyukova (1969) assigned the dark-unicolored black porgy to *A. schlegelii schlegelii* and took the striped specimens as *A. schlegelii czerskii*. However, due to morphology similarity, the phylogenetic relationship between the two subspecies remains unclear. They were either classified in a single species (Akazaki, 1962; Parin, 2003) or in separate species (Dolganov et al., 2008) or subspecies (Lindberg and Krasyukova, 1969; FAO Fishbase, 2010). In this study, the findings of mtDNA identification in the two subspecies could provide convincing evidences that they belong to the same species.

Ward et al. (2005) compared the level of COI sequence divergence within species, genera, families, orders, and classes for Australian marine fishes, and suggested that the minimum genetic distance between species should be 0.02. On the other hand, data from some literatures suggested that the intra-generic genetic distance in marine fishes by CR sequence should be 0.060 to 0.247 (Chen et al., 1998; Kong et al., 2007; Shao et al., 2007), although the minimum genetic distance of species identification is not defined in this sequence. In this study, the mean genetic distance between *A. schlegelii schlegelii* and *A. schlegelii czerskii* was 0.0015 in COI and 0.0051 in CR, respectively, which are much less than those among other the three species of *Acanthopagrus* (0.0667 to 0.0954 in COI and 0.2267 to 0.2480 in CR) (Table 2). The values are also lower than those among populations in *Engraulis japonicus* (0.0064 in COI) (Yu et al., 2005), *Chelon haematocheilus* (0.0155 to 0.0241 in CR) (Liu et al., 2007) and *Pennahia argentata* (0.030 in CR) (Han et al., 2008). Moreover, haplotypes of *A. schlegelii schlegelii* and *A. schlegelii czerskii* did not form reciprocal monophyletic clades in the phylogenetic trees for both mitochondrial genes. Therefore, we concluded that *A. schlegelii schlegelii* and *A. schlegelii czerskii* should be classified into the same species: *A. schlegelii*.

In this study, two color patterns in the black porgy co-occurred in the Chinese coastal waters from the Yellow Sea to East China Sea (Table 1), which indicates that *A. schlegelii schlegelii* and *A. schlegelii czerskii* are not two valid subspecies. This is consistent with the viewpoint of Dolganov et al. (2008). Subsequently, the Czersky black porgy *S. swinhonis czerskii* (=*A. schlegelii czerskii*) was thought to be a junior subjective synonym of *A. schlegelii* (Kharin and Markevich, 2010). Furthermore, Parin (2003) ascribed *Chrysophrys swinhonis, Sparus swinhonis czerskii, S. macrocephalus czerskii* and *A. schlegelii czerskii* to the synonyms of *A. schlegelii*. These findings together with molecular analysis results from this study reveal that *A. schlegelii schlegelii* and *A. schlegelii czerskii* are the subjective synonyms of *A. schlegelii*.

Sparus macrocephalus was first described by Basilewsky (1855) (type locality: Bohai Bay, China). This species is similar to *A. schlegelii czerskii* in overall appearance, but the teeth in jaws wholly match the diagnostic traits of the genus *Pagrus*. Thereby, Lindberg and Krasyukova (1969) introduced the name *Sparus macrocephalus* Basilewsky into the synonym of *Pagrus major*, and this was also proposed by Iwatsuki and Carpenter (2006). In the previous studies, Chinese scientists regarded all the black porgy with striped coloration and five and a half scales above the lateral line as *Sparus macrocephalus* (not of Basilewsky, 1855) (Zhu et al., 1962, 1963; Cheng et al., 1987), which was a synonyms of *A. schlegelii*.

Akazaki (1962) assumed that the striped specimens (*A. schlegelii czerskii*) are the juvenile of *A. schlegelii*, and the dark-unicolored specimens (*A. schlegelii schlegelii*) are mature individuals of *A. schlegelii*. Nevertheless, a striped specimen of *A. schlegelii* with a standard length of 500 mm (mature individual) was reported in Japan (Nakabo, 2002). In our study, the two body color patterns were observed in both juvenile and mature individuals (Table 1).

In conclusion, the evidences from morphological and molecular genetic studies indicate that the two morphologically different black porgies, which are sympatric in the coastal waters of the Northwest Pacific Ocean, belong to the same species *A. schlegelii*. As discussed previously, the taxonomic classification and phylogenetic relationship of *A. schlegelii* is controversial because of variable body color and wide distribution. Further investigation is needed to study the relationship between the different color patterns and ecological adaptation in this fish species.

ACKNOWLEDGMENTS

This research was financially supported by the National Natural Science Foundation of China (Grant No.30970317, 41006084, 31061160187), Natural Science Foundation of Guangdong Province of China (Grant No.10152408801000013), Foundation for Distinguished Young Talents in Higher Education of Guangdong, China (LYM10088), Open Foundation from Ocean Fishery Science and Technology in yhe most Important Subjects of Zhejiang (20100116), and Foundation of Guangdong Ocean University for Talent Introduction Professor Wan-Shu Hong is thanked for his assistance with the English.

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