THE RAFFLES BULLETIN OF ZOOLOGY 2008 Supplement No. **19**: 215–223
Date of Publication: 15 Dec.2008

MOLECULAR EVIDENCE FOR GENETIC DIFFERENTATION OF THE *OPSARIICHTHYS BIDENS* COMPLEX (TELEOSTEI: CYPRINIDAE) IN SOUTHERN CHINA AROUND SOUTH CHINA SEA AND THE VALIDITY OF *OPSARIICTHYS HAINANENSIS*

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***ABSTRACT.* –** The widely-distributed cyprinid, *Opsariithchys bidens* Günther, 1873, from Hainan Island and mainland southern China, northern South China Sea is revised on the basis of genetic differentiation of mitogenetic markers and other characters. Molecular phylogenetic analyses carried out using the mtDNA D-loop sequences for members of the *Opsariithchys bidens* species complex from different basins in Hainan have revealed that great mitogenetic differentiation of current endemic species exists and that these species are not of the same lineage as that of typical *Opsariithchys bidens* from mainland China. The valid name for the endemic species from Hainan should be *Opsariichthys hainanensis* Nichols & Pope, 1927, which has been ignored since its original description. *Opsariichthys hainanensis* is redescribed.

***KEY WORDS*. –** *Opsariichthys bidens*, *Opsariichthys hainanensis*, molecular evolution, fish taxonomy, South China Sea.

INTRODUCTION

The cyprinid fishes are the most important members of the freshwater fish fauna in mainland China and Taiwan (Chen, 1998; Chen & Fang, 1999). One of the genera, *Opsariichthys* Bleeker, 1873, was initially described as *Leuciscus uncirostris* Temminck & Schlegel, 1846. It is a common and widely distributed species ranging from Japan, via Korea, China to Vietnam (Chen, 1998; Kawanabe et al., 2002). In Japanese and Korean waters, there is only one species, *Opsariitchthys uncirostris*. In mainland China, another widely distributed species can be recognized, *Opsariichthys bidens* Günther, 1873 (Chen, 1998). However, detailed surveys of different river basins have suggested that higher diversity exists than is presently known and recorded for many groups of freshwater fishes, especially the cyprinids and gobies (Chen et al., 1999; Chen et al, 2002; Chen & Fang, 2006).

Molecular sequence analyses have provided good resolution for recognizing freshwater gobiid species diversity and in providing insights into their phylogeny in river basins of Hainan island (Chen et al., 2002) and also for the stripe minnow, *Candidia*, from Taiwan (Wu et al., 2007). The aim of this study is use both molecular and morphological approaches to establish if the fishes collected from Hainan island belong to same species, the so-called common species *Opsariichthys bidens* or if they belong to another, discrete species.

MATERIALS AND METHODS

***Sample collection.* –** All cyprinid fishes of the genus *Opsariichthys* and related outgroups were collected by either cast-neting or electro-fishing. Specimens and the partial fin tissues used for molecular analysis were directly preserved in 95% ethanol when caught and transferred frozen after preservation to the laboratory. Specimens used

for morphological studies were fixed in 10% formalin before being transferred into 70% ethanol for long-term preservation.

***Molecular phylogenetic analysis.* –** All DNA extractions of the cyprinids were carried out according to the general protocols of the Phenol-chloroform method (Sambrook et al., 1989; Chen et al., 1998, 2002). The DNA fragment of about 1200 bp, including the full length of the D-loop region, were amplified by polymerase chain reaction (PCR) using primers based on the flanking region (CYP-THRA: 5'–AAAGCATCGGTCTTGTAATCCGAAG–3'; CYP-12SB: 5'–CATGCGGAGTTTCTTAGGTC–3') that were designed from the conserved sequences of tRNA-PHE and 12S rRNA (Chen et al., 1998, 2002; Chen & Chang, 2007; Wu et al., 2007). PCR was done in a MODEL 2700 or 9700 thermal cycler (Perkin-Elmer) and 30–40 cycles were carried out. The 25 µL reaction volume contained 14.4 µL of sterile distilled water, 2.5 µL of 10× PCR buffer (Takara), 2.0 µL of dNTP (2.5 mM each), 2.5 µL of each primer, 0.1 µL of 0.5 unit Ex *Taq* (Takara) and 10 µL of template. The thermal cycler profile was as follows: denaturation at 94°C for 15 seconds, annealing at 50°C for 15 seconds and extension at 72°C for 60 seconds. A negative control without template was carried out for each run of PCR. The PCR products were run on a 1.0% L 03 agarose gel (Takara) and stained with ethidium bromide for band characterization under ultraviolet trans-illumination.

Double-stranded PCR products were purified using a kit (Roche, High Pure Product Purification kit), before undergoing direct cycle sequencing with dye-labeled terminators (ABI Big-Dye kit). The sequencing primers used were either same as those for PCR or following two primers: CYP-DLMF1: 5'–CATGCGGAGTTTCTTGTC–3' and CYP-DLMR2: 5'–GCTCGGCATGTTGGGTAA–3'. All sequencing reactions were performed according to the manufacturer's instructions. Labeled fragments were analyzed using as ABI PRISM Model 377-64 DNA Automated sequencer (ABI).

Nucleotide sequence alignment was verified manually after running through CLUSTAL W (Thompson et al., 1994) and BIOEDIT version 5.9 (Hall, 2001). The analysis of aligned mutation sites were conducted using Molecular Evolutionary Genetics Analysis (MEGA) version 3 (Kumar et al., 2004) for aligned mutation sites analysis.

The parsimony (MP) analysis was carried out using PAUP* version 4.0B10 (Swofford, 2003) using heuristic search. Branch support was established via bootstrap analysis (2,000 replications). For the Bayesian (BI) analysis, the best-fitting model for sequence evolution was determined for mtDNA D-loop sequences using MrMODELTEST version 2.2 (Nylander, 2005). The BI analyses were performed using MrBayes 3.0 (Ronquist & Huelsenbeck, 2003). The posterior probabilities of each node were computed from remaining 75% of all sampled trees.

***Morphological studies.* –** The meristic counts and morphometric measurements generally follow Hosoya et al. (2003). The specimens used in this current research have been deposited in the National Museum of Natural Science, Taichung (NMNS) and the Institute of Marine Biology, National Taiwan Ocean University, Keelung (NTOU).

RESULTS

***Molecular phylogenetic analysis.* –** The complete D-loop sequences were amplified and sequenced for the different species groups of *Opsariichthys* from southern China and Japan (Table 1). Among them, the total lengths of complete D-loop sequences of *Opsariichthys* species were found to be from 922–927 bp. The shortest sequences, 922 bp, were seen in *Opsariichthys* sp. from the Pearl River basin, and the longest was 925 bp, obtained from *Opsariichthys hainanensis* from Hainan Island. The length of aligned sequence for those different OTUs with outgroup comparison of *Zacco platypus* were up to 933 bp and with 163 divergent sites (Appendix I).

Based on the outgroup assignment of *Zacco platypus*, the phylogenetic analysis of both MP and BI methods provided congruent results at the species level (Figs. 1 & 2). In MP analysis, 160 trees with equal minimum tree length 152 were obtained, with CI = 0.8158 and RI = 0.9561. The consensus tree indicates four monophyletic groups with high. bootstrap support: Japanese *O. uncirostris*; the three Chinese species *O. bidens*, *O. hainanensis* and *Opsariichthys* sp. (Fig. 1). It suggests that *O. hainanensis* maybe a basal taxon within this genus. In the BI analysis, the phylogenetic tree reconstructed by using HKY85+G model and the result appears to be congruent with MP phylogeny, which supports the monophyly of *O. uncirostris*, *O. bidens* and *Opsariichthys* sp. (Fig. 2). and shows a distinct, separate taxon, *O. hainanensi*s. All specific level of nodes are with posterior probabilities as high as 91–100. Two species from mainland China, *O. bidens* and *O*. sp., are grouped as the sister species.

According to the p-distance among different species of *Opsariichthys* re-examined here, the low genetic divergence of 2.1–2.9% was seen between *O. uncirostris* and *Opsariichthys* sp. A higher genetic divergence of 6.1–7.2% seen between *O. bidens* and *O*. *hainanensis*.

It was interesting to discover different haplotypes of *O. hainanensis* from the different basins of Hainan Island. These were distinct from each other and from those fish from the Changhuajiang and Tengchiaotung River basins.

Among them, four major mitogenetic groups of *O. hainanensis* were detected. They consist of the Nandujiang basin group; the Wangchuang River basin group; the Linshui River basin group and the the Changhuajiang-Tengchiaotung River basin group. The highest genetic divergence of 0.9-1.1% was seen between the Nandujiang and Linshui River basin groups. The overall divergence may reveal that the possibility of long separation of the Linshui River basin from the northern and western basins.

SYSTEMATICS

Opsariichthys bidens Günther, 1873
(Fig. 3)

Opsariichthys bidens Günther, 1873: 249 (Shanghai City, China); Chen, 1998: 47 (in part).
Opsariichthys chekianensis Shaw, 1930: 113 (Zhejiang Province, China).

Material examined. – NTOU P-2008-06-381, 6 ex., 69.7–84.8 mm SL, Tachiao Market, Fenghua City, Zhejiang Province, China, Coll. J. H. Wu & S. P. Huang, 10 Jan.2008. NTOU P-2008-06-389, 15 ex., 112.9–163.2 mm SL, Central Market, Xianju County, Zhejiang Province, China, Coll. J. H. Wu & S. P. Huang, 8 Jan.2008. NMNSF01648, 64.3 mm SL, Changhua Town, Linan City, Zhejiang Province, China, Coll. N. H. Jang-Liaw, 16 Aug.2006.

Diagnosis. – This species is distinguished from its congeners by the following unique combination of features: dorsal fin rays iii, 7; anal fin rays iii, 8–9 (modally 9); pectoral fin rays i, 14–15 (modally 15); lateral-line scales 45–46; the anterior scale series to vertical of ventral fin origin 15; pre-dorsal scales 16–17 (modally 17); upper scale rows above lateral-line 9; scale rows below lateral-line 3; gill-rakers 10–11; total vertebrae 41–42; lower jaw prominent, anterior lateral of upper jaw with a very distinctly deep notch; maxillary extending to midline of orbit in adult; body rather elongate, body depth about 28.7% in male and 18.8–21.1% in female; body silver white in lateral and yellowish brown in dorsal; and snout tip orange red in female.

Opsariichthys hainanensis Nichols & Pope, 1927
(Figs. 3 & 4)

Opsariichthys hainanensis Nichols & Pope, 1927: 367 (Noda, Hainan Island, China).

Materials examined. – NTOU P-2008-6-382, 10 ex., 39.7–71.7 mm SL, Tengchiaotung River, Pouting County, Hainan Province, China, Coll. I-S. Chen & S. P. Huang, 9 Sep.2005; NTOU P-2008-6-383, 2 ex., 46.8–54.3 mm SL, Partsun, Linshui River basin, Pouting County, Hainan Province, China, Coll. I-S. Chen & S. P. Huang, 10 Sep.2005; NTOU P-2008-6-384, 2 ex., 54.4–71.7 mm SL, Linshui River basin, Linshui County, Hainan Province, China,

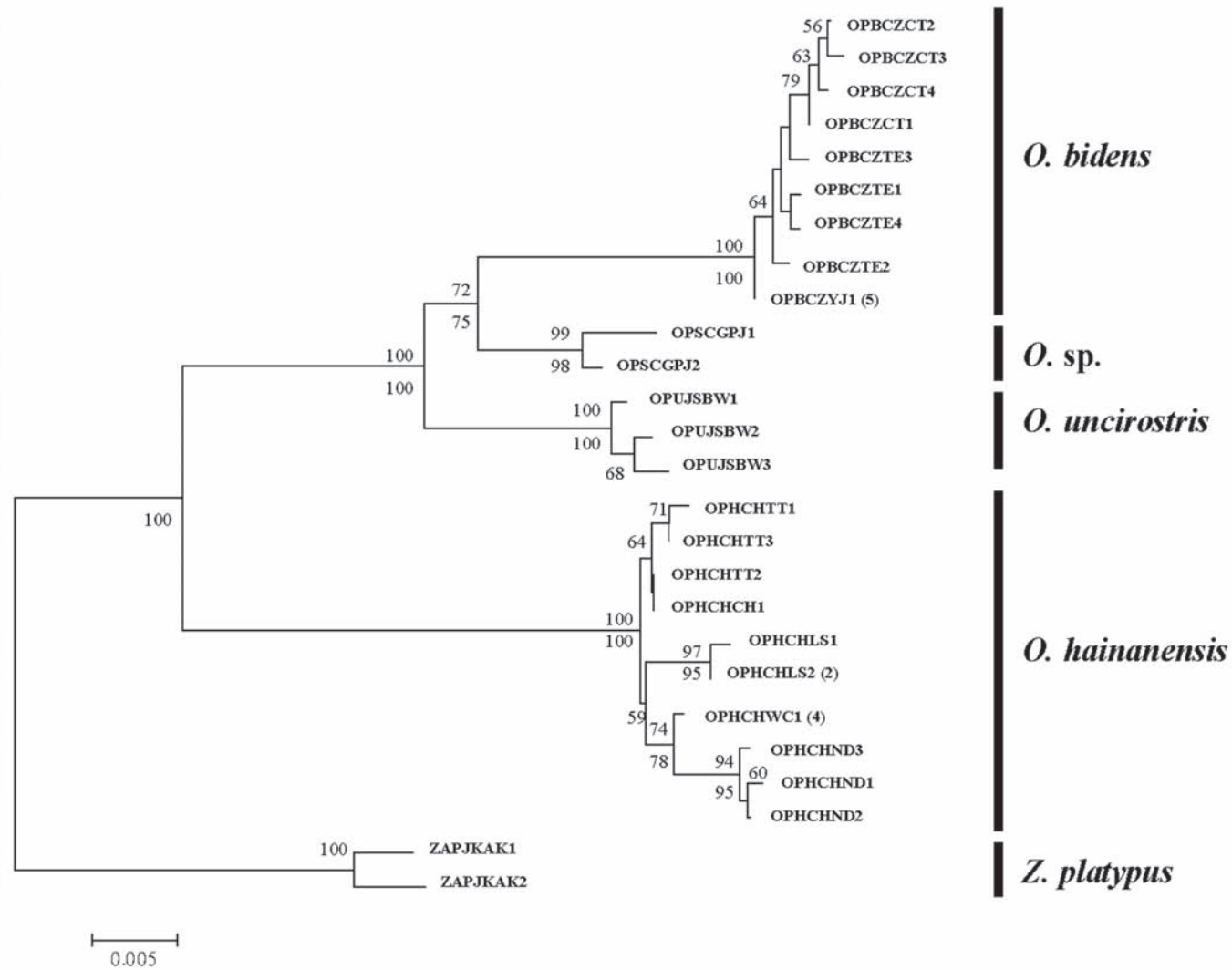

Fig. 1. Molecular phylogenetic tree of *Opsariichthys* species from China and Japan reconstructed by neighbouring-joining method based on the HKY85+G model (value above the branch: bootstrap number, 1000 replications). The similar topology for bootstrap consensus tree by maximum parsimony method list only the bootstrap (values below the branch: bootstrap number, 1000 replications).

Coll. I-S. Chen & S. P. Huang, 10 Sep.2005; NTOU P-2008-6-385, 2 ex., 54.1–65.5 mm SL, Leunan, Nandujiang basin, Chungjung County, Hainan Province, China, Coll. I-S. Chen & S. P. Huang, 11 Sep.2005; NTOU P-2008-6-386, 57.2 mm SL, Nankai, Nandujiang basin, Paishar County, Hainan Province, China, Coll. I-S. Chen & S. P. Huang, 12 Sep.2005; NTOU P-2008-6-387, 8 ex., 54.8–63.2 mm SL, Wangchuang River basin, Chungjung County, Hainan Province, China, Coll. I-S. Chen & S. P. Huang, 11 Sep.2005.

***Diagnosis.* –** This species is distinguished from its congeners by following unique combination of features: dorsal fin rays iii, 7–8 (modally 7); anal fin rays iii, 9–10 (modally 9); pectoral fin rays i, 13–15 (modally 14); lateral-line scales 40–41; the anterior scale series to vertical of ventral fin origin 13–14; predorsal scales 16–17 (modally 17); upper scale rows above lateral-line 8–9 (modally 9); scale rows below lateral-line 4; gill-rakers 10–11; total vertebrae 39–41 (modally 40); lower jaw prominent, anterior tip of upper jaw with a deep notch; maxillary extending to midline of orbit in adult; body rather fusi-form, body depth 27.5–30.9% in male, body with 9–11 vertical greenish blue stripes in male; round tubercles on lower jaw rather large, as 2 rows in male.

***Redescription.* –** Dorsal fin rays iii, 7–8 (modally 7); anal fin rays iii, 9–10 (modally 9); pectoral fin rays i, 13–15 (modally 14); lateral-line scales 40–41; the anterior scale series to vertical of ventral fin origin 13–14; predorsal scales 16–17 (modally 17); upper scale rows above lateral-line 8–9 (modally 9); scale rows below lateral-line 4; gill-rakers 10–11; total vertebrae 39–41 (modally 40) (distribution frequency of meristic features listed in Tables 2, 3).

Morphometric data listed in Table 4. Body rather high, fusiform and compressed. Head rather large, lower jaw prominent, anterior tip of upper jaw possessing a distinct notch. No maxillary barbels. Body covered cycloid scales. Lateral-line complete, extending downward from the upper area of gill opening along lower half of body to caudal fin base.

Large tubercles on both jaws and cheek, with two rows of large ones on lower jaws in male, and rather small tubercles in female. Pectoral fin extending beyond ventral fin origin when depressed in male, but not reaching the origin in female. Ventral fin long, extending beyond anal fin origin in male, but not reaching the origin in female. Anal fin rays

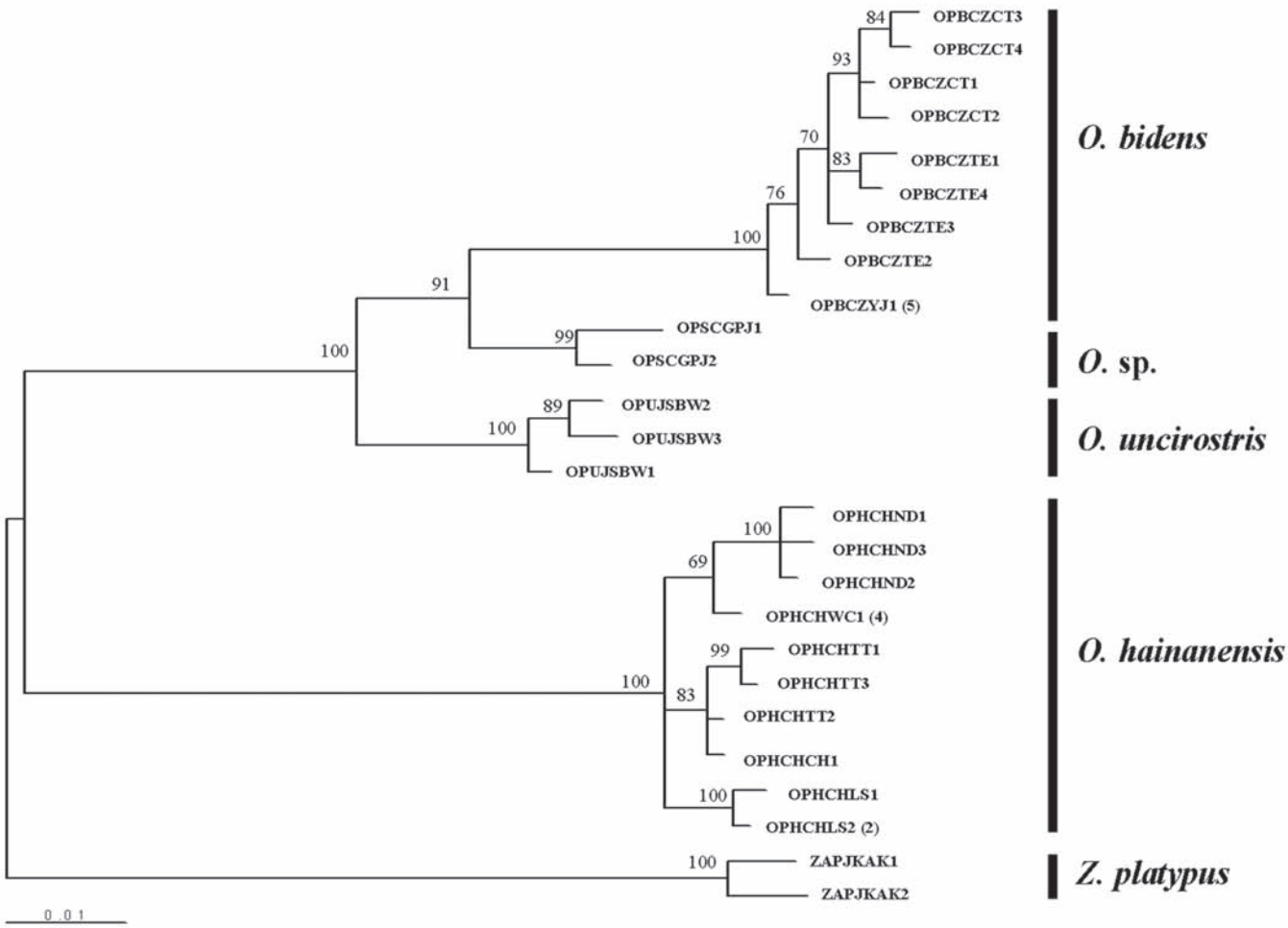

Fig. 2. Molecular phylogenetic tree of *Opsariichthys* species from China and Japan reconstructed by Bayesian analysis based on the HKY85+G model (values above the branch: posterior probability).

Table 1. Sampling localities and OTU codes of molecular sequence analysis of *Opsariichthys* species from China and Japan.

Code	Species name	Locality
OPUJSBW1	*Opsariichthys uncirostris*	Lake Biwa, Otsu, Honshu, Japan
OPUJSBW2	*Opsariichthys uncirostris*	Lake Biwa, Otsu, Honshu, Japan
OPUJSBW3	*Opsariichthys uncirostris*	Lake Biwa, Otsu, Honshu, Japan
OPBCZCT1	*Opsariichthys bidens*	Chientangjiang, Zhejiang Province, China
OPBCZCT2	*Opsariichthys bidens*	Chientangjiang, Zhejiang Province, China
OPBCZCT3	*Opsariichthys bidens*	Chientangjiang, Zhejiang Province, China
OPBCZCT4	*Opsariichthys bidens*	Chientangjiang, Zhejiang Province, China
OPBCZTE1	*Opsariichthys bidens*	Tsaoeojiang, Zhejiang Province, China
OPBCZTE2	*Opsariichthys bidens*	Tsaoeojiang, Zhejiang Province, China
OPBCZTE3	*Opsariichthys bidens*	Tsaoeojiang, Zhejiang Province, China
OPBCZTE4	*Opsariichthys bidens*	Tsaoeojiang, Zhejiang Province, China
OPBCZYJ1	*Opsariichthys bidens*	Tsaoeojiang, Zhejiang Province, China
OPHCHCH1	*Opsariichthys hainanensis*	Changhuajiang, Hainan Island, China
OPHCHND1	*Opsariichthys hainanensis*	Nandujiang, Hainan Island, China
OPHCHND2	*Opsariichthys hainanensis*	Nandujiang, Hainan Island, China
OPHCHND3	*Opsariichthys hainanensis*	Nandujiang, Hainan Island, China
OPHCHLS1	*Opsariichthys hainanensis*	Linshui River, Hainan Island, China
OPHCHLS2	*Opsariichthys hainanensis*	Linshui River, Hainan Island, China
OPHCHTT1	*Opsariichthys hainanensis*	Tengchiaotung River, Hainan Island, China
OPHCHTT2	*Opsariichthys hainanensis*	Tengchiaotung River, Hainan Island, China
OPHCHTT3	*Opsariichthys hainanensis*	Tengchiaotung River, Hainan Island, China
OPHCHWC1	*Opsariichthys hainanensis*	Wangchuang River, Hainan Island, China
OPSCGPJ1	*Opsariichthys* sp.	Pearl River, Guangdong Province, China
OPSCGPJ2	*Opsariichthys* sp.	Pearl River, Guangdong Province, China
ZAPJKAK1	*Zacco platypus*	Kagoshima-ken, Japan
ZAPJKAK2	*Zacco platypus*	Kagoshima-ken, Japan

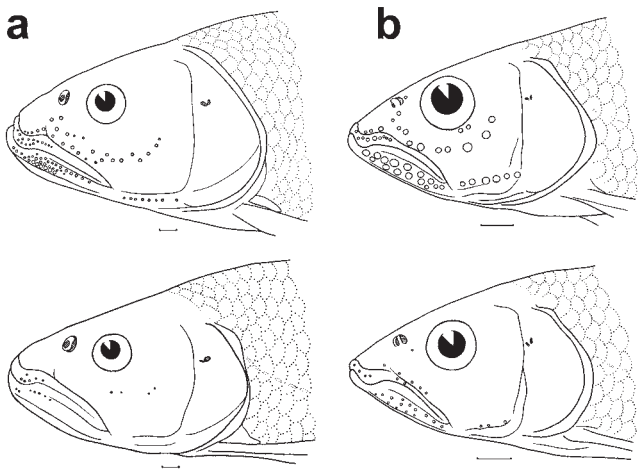

Fig. 3. Head features of a, *Opsariichthys bidens*, male (upper one) NTOU P-2008-06-389, 155.1 mm SL, female (lower one) NTOU P-2008-06-389, 139.4 mm SL, central market, Xianjju County, Zhejiang Province, China; b, *Opsariichthys hainanensis*, male (upper one) NTOU P-2008-06-385, 65.5 mm SL, Nandujiang, Hainan Island, China; female (lower one) NTOU P-2008-06-382, 69.1 mm SL, Tengchiaotung River, Hainan Island, China. Scale bars = 3 mm.

Fig. 4. Specimen photos of *Opsariichthys hainanensis*: a, male, NTOU P-2008-06-385, 65.5 mm SL, Nandujiang, Hainan Island, China; b, female, NTOU P-2008-06-387, 61.4 mm SL, Wangchuang River, Hainan Island, China

Table 2. Frequency distribution of meristic counts of population of *Opsariichthys* species in southern China.

	D iii			A iii				P1 i				P2 i		
	7	8	Av	8	9	10	Av	13	14	15	Av	7	8	Av
Opsariichthys bidens	7	–	7.0	1	6	–	8.9	–	4	4	14.5	–	14	7.0
Opsariichthys hainanensis	20	2	7.1	–	19	3	9.1	4	21	13	14.2	6	38	7.9

	Predorsal scales							Lateral line scales								Scales above lateral line to dorsal fin origin		
	16	17	18	19	20	21	Av	40	41	42	43	44	45	46	Av	8	9	Av
Opsariichthys bidens	–	–	–	1	5	1	20.0	–	–	–	–	–	10	4	45.3	–	7	9.0
Opsariichthys hainanensis	9	13	–	–	–	–	16.6	31	13	–	–	–	–	–	40.3	1	21	9.0

	Scales below lateral line to anal fin origin			Number of total vertebrae				
	3	4	Av	39	40	41	42	Av
Opsariichthys bidens	6	1	3.1			3	3	41.5
Opsariichthys hainanensis	–	22	4.0	2	14	1	–	39.9

Table 3. Frequency distribution of meristic counts of three *Opsariichthys hainanensis* from different river basins of Hainan, China.

	D iii			A iii			P1 i				P2 i		
	7	8	Av	9	10	Av	13	14	15	Av	7	8	Av
Nandujiang	3	–	7.0	3	–	9.0	–	4	1	14.2	1	5	7.8
Wangchuang River	8	–	7.0	8	–	9.0	–	4	12	14.8	2	14	7.9
Tengchiaotung River	5	2	7.3	5	2	9.3	4	7	–	13.6	3	11	7.8
Linshui River	4	–	7.0	3	1	9.3	–	6	–	14.0	–	8	8.0

	Predorsal scales			Lateral line scales			Scales above lateral line to dorsal fin origin			Scales below lateral line to anal fin origin		Number of total vertebrae			
	16	17	Av	40	41	Av	8	9	Av	4	Av	39	40	41	Av
Nandujiang	3	–	16.0	5	1	40.2	1	2	8.7	3	4.0	–	3	–	40.0
Wangchuang River	3	5	16.6	9	7	40.4	–	8	9.0	8	4.0	1	5	–	39.8
Tengchiaotung River	2	5	16.7	13	1	40.1	–	7	9.0	7	4.0	1	2	1	40.0
Linshui River	1	3	16.8	4	4	40.5	–	4	9.0	4	4.0	–	4	–	40.0

rather elongate especially first to third branched rays longer in male, the rear tip extending beyond vertical line of caudal fin base. Caudal fin with slightly longer lower lobe.

Body silver white in lateral and yellowish brown in dorsal area. Body with 9–11 vertical greenish blue stripes with unequal widths in male and indistinct in female. Tubercles larger which larger ones about equal to nasal diameter in male, and smaller in female. Round tubercles on lower jaw rather large as 2 rows in male. All fins pale white to yellowish.

Distribution. **–** *Opsariichthys hainanensis* is found from the all different basins (Nandujiang, Wangchuang River, Linshui River, and Tengchiaotung River basins) in Hainan Island, China. It may be a cyprinid species endemic to Hainan island.

Habitat. **–** *Opsariichthys hainanensis* prefers the tributaries of river basins with muddy, sandy substrata with pebbles under moderate or slow water flow.

Remarks. **–** The features shared between both *Opsariichthys hainanensis* and *Opsariichthys bidens* include the distinct deep notch of the upper jaw and similar fin rays counts. *Opsariichthys hainanensis* can be distinguished from *O. bidens* by the following combination of features: (1) lower counts of lateral-line scales 40–41 (vs. 45–46); (2) lower counts of predorsal scales 16–17 (vs. 19–20); (3) higher counts of scale rows below lateral-line scales modally 4 (vs.

Table 4. Morphometry of two *Opsariichthys* species in southern China.

	Opsariichthys bidens		*Opsariichthys hainanensis*	
	Male	Female	Male	Female
	1	6	3	14
As % of SL				
Head length	25.3	23.4–25.4 (24.4)	26.5–28.6 (27.3)	24.0–26.8 (25.6)
Body depth	28.7	18.8–21.1 (19.7)	27.6–30.9 (29.1)	22.4–27.8 (25.0)
Body width in dorsal origin	13.7	10.5–11.7 (11.2)	12.3–14.9 (13.7)	11.2–14.2 (12.2)
Body width in anal origin	10.2	8.2–9.7 (8.8)	9.5–11.8 (10.6)	7.2–9.6 (8.6)
Depth of caudal peduncle	10.1	8.8–9.5 (9.1)	10.2–10.9 (10.5)	9.3–10.6 (10.0)
Length of caudal peduncle	20.7	18.7–21.0 (19.9)	17.9–19.7 (18.6)	17.9–20.3 (18.9)
Predorsal length	50.9	49.5–52.4 (50.8)	49.0–51.9 (50.6)	48.3–52.8 (50.6)
Preanal length	70.7	69.2–72.4 (71.2)	68.4–70.3 (69.1)	68.3–72.5 (70.6)
Preventral length	54.4	52.6–55.7 (54.1)	53.3–55.8 (54.4)	51.2–56.5 (53.4)
Dorsal origin to caudal base	52.7	48.3–51.0 (49.7)	50.9–52.5 (51.9)	50.2–53.5 (51.9)
Pectoral origin to pelvic insertion	26.0	25.3–27.9 (26.3)	26.1–28.3 (27.1)	25.1–28.8 (26.9)
Length of longest dorsal ray	23.0	18.0–19.0 (18.6)	21.4–22.8 (22.3)	15.7–18.8 (17.6)
Length of longest anal ray	29.1	18.6–23.7 (21.3)	24.9–25.7 (25.3)	14.3–22.7 (17.3)
Length of longest pectoral ray	21.9	18.0–20.3 (19.0)	24.1–25.8 (25.0)	17.6–21.8 (19.7)
Length of dorsal fin base	12.0	9.9–11.4 (10.6)	12.2–14.0 (13.3)	10.7–13.3 (12.1)
Length of anal fin base	13.1	10.8–11.8 (11.3)	14.4–15.7 (14.9)	12.2–15.3 (13.1)
As % of HL				
Head width in nasal section	53.1	47.2–51.3(49.4)	50.5–53.7(52.2)	47.3–55.3(51.0)
Snout length	44.6	38.5–40.8(39.8)	37.7–38.1(37.9)	36.5–38.9(37.8)
Inter–orbital width	39.6	34.3–36.2(35.0)	32.4–32.8(32.9)	30.1–35.3(32.0)
Orbit diameter	21.1	26.8–27.9(27.4)	25.3–26.6(25.8)	26.1–29.8(28.1)
Upper jaw length	60.9	54.2–58.8(56.5)	53.2–54.9(54.3)	53.3–57.5(55.7)

3); (4) higher body depth in female 22.4–27.8% (vs. 18.8–21.1%); (5) short snout length 36.5–38.9% (vs. 39.4–40.8%); and (6) two rows of below large tubercles lower jaw in male (vs. three to four rows of tiny tubercles in male). Although this study did not include the re-examination of the holotype of *Opsariichthys hainanensis*, our data of collections from different basins of Hainan island match well with the original description by Nichols & Pope (1927).

***Comparative material. – Opsariichthys* sp.** NMNS-uncat., 5 ex., 53.2–68.5 mm SL, Fengjen Town, Longtsuan County, the Pearl River basin, Guangdong Province, China, Coll. N.H. Jang-Liaw & W.H. Chou, 18 Nov.2007.

DISCUSSION

This comparative study combining both morphological and molecular approaches indicates the validity of *O. hainanensis* which is distinguishable from *O. bidens* from Eastern China. The genetic divergence (p-distance) of D-loop sequences of 6.1–7.2% between *O. bidens* and *O. hainanensis* is even higher than the 5.8–6.0% between Japanese *Nipponocypris temmickii* (Temminck & Schlegel, 1846) and *Nipponocypris sieboldii* (Temminck & Schlegel, 1846) and the 3.5–3.6% between two sibling species of Taiwanese *Candidia barbatus* (Regan, 1908) and *Candidia pingtungensis* Chen et al, 2008. The distinct separation between such allopatric, sibling species group in southern China is also clearly apparent in the morphological comparison of both meristic and body-proportion data as well as body coloration pattern.

In recent years, Nguyen & Nguyen (2000) documented further more species of *Opsariichthys* from Vietnam when they described *O. dienbienensis* and *O. songmaenesis*. These reveal the possibility of sympatric species occurring in a tropical Vietnamese river basin. Although all Chinese species need to be re-examined to resolve any synonymies, the stability of nomenclature is unproblematic as *O. hainanensis* (as the Hainanese *Opsariichthys* species) has nomenclatural priority. In the Pearl River basin, *O.* sp. seems to be a rather discrete species distinguished form both *O. bidens* and *O. hainanensis*, and even the species is highly possible to be undescribed, but it is still needed the further more comparisons of adult specimens to clarify its suitable specific name. Therefore, the nomenclatural validity of recent published Vietnamese species requires verification. Based on this phylogenetic analysis, we also suggest that the basal clade of *Opsariichthys* species should occur either in southern China or in adjacent Country, Vietnam. Further analysis, including specimens from Vietnam, will be required.

ACKNOWLEDGEMENTS

The first author (ISC) is very grateful for grants from the National Science Council & Agriculture Council, Taipei, Taiwan (2007 and 2008) in support of this research in Taiwan. Our work has also been partially supported by CMBB in NTOU.

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Appendix I. Divergent sites of aligned completed D-loop sequences (length: 933 bp) of *Opsariichthys* from China and Japan.

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            000000000001111111111111111111122222222222222222333333333444444444455555555555555
            122235556990111112244456666788880111122223334677835688899001123456800013333356666 6
            635860581456124890212390156505781158934784670802601801309241481753423900145670124 5
OPUJSBW1    GAATTCGCTCCCTTTACGAGACTTCCTGATACCTTCCCTAGACGATGTCACGGATCCTCAGACAACTTCTCATTTTCGCTAA
OPUJSBW2    ..........T.....................................................T.....................
OPUJSBW3    ..........................................................T.....T.....................
OPBCZTE1    ...C....C.........GA.T..T.C..C.T.C.....G........T..TAG.TT....G.C...CT.T...C.......
OPBCZTE2    ...C....C.........GA.T..T.C..C.T.C.....G........T..TAG.TT....G.C...CT.T...C.......
OPBCZTE3    .G.C....C.........GA.T..T.C..C.T.C.....G........T..TAG.TT....G.C...CT.T...C.......
OPBCZTE4    .G.C....C.........GA.T..T.C..C.T.C.....G........T..TAG.TT....G.C...CT.T...C.......
OPBCZYJ1    ...C..............GA.T..T.C..C.T.C.....G........T..TAG.TT....G.C...CT.T...C.......
OPBCZCT1    .G.C....C.........GA.T..T.C..C.T.C.....G........T..TAGCTT....G.C...CT.T...C.......
OPBCZCT2    .G.C....C.........GA.T..T.C..C.T.C.....G........T..TAGCTT....G.C...CT.T...C.......
OPBCZCT3    .GTC....C.........GA.T..T.C..C.T.C.....G........T..TAGCTT....G.C...CT.T...C.......
OPBCZCT4    .GTC....C.........GA.T..T.C..C.T.C.....G........T..TAGCTT....G.C...CT.T...C.......
OPSCGPJ1    ...C..............GA.T.CT......T..C....GA.......................G...T.....A.......
OPSCGPJ2    ...C..............GA.T.CT......T.......GA.......................G...T.....A.......
OPHCHWC1    ....AT......A......ACTC.TA.....T...TTTAGACTA........A...T.............TG....TAGCCC
OPHCHND1    ....AT......A......ACTC.TA.....T...TTTAGACTA........A...T.............TG....TAGCCC
OPHCHND2    ....AT......A......ACTC.TA.....T...TTTAGACTA........A...T.............TG....TAGCCC
OPHCHND3    ....AT......A......ACTC.TA.....T...TTTAGACTA........A...T...........T.TG....TAGCCC
OPHCHLS1    ....AT......A......ACT..TA.....T...TTTAGATTA........AG..T.........C...TG....TAGCCC
OPHCHLS2    ....AT......A......ACT..TA.....T...TTTAGATTA........AG..T.........C...TG....TAGCCC
OPHCHTT1    ....AT......A......ACT..TA.....T...TTTAGATTA.....G..A...T.............TG....TAG.CC
OPHCHTT2    ....AT......A......ACT..TA.....T...TTTAGATTA........A...T.............TG....TAG.CC
OPHCHTT3    ....AT......A......ACT..TA.....T...TTTAGATTA........A...T.............TG....TAG.CC
OPHCHCH1    ....AT......A......ACT..TA.....T...TTTAGATTA........A...T.............TG....TAG.CC
PASCGPJ1    A...A.AT.TT.AACGTA.A....A.CTT.G.G.AAA.....TACCACT.TTA..A.CTTAG.CGT.CTCT.AA.C.AG...
PASCGPJ2    A...A.AT.TT.AACGTA.A....A.CTT.G.G.AAA.....TACCACT.TTA..A.CTTAG.CGT.CTCT.AA.C.AG...

            555556666666666666777777777777777777888888888888888888888888888888888999999999999
            666780133455578888112445567777789999334444555666666677777888888888999900000111222 2
            678889926701270128577483891237990349094589012045678912789012345789467834678256125
OPUJSBW1    TAATTGTTCCTCTCCACTGACGGGTAAAGGAGAGCTTGTTTTTAACAATGACCATCTCTATTGACTGATAGTAAATGAGAA
OPUJSBW2    ..G.....................................................................................
OPUJSBW3    ..G.....................................................................T...............
OPBCZTE1    ....C.............A......G.................T......G.T........G..........G......G.
OPBCZTE2    ....C.............A......G.....................G.G.T.....................G......G.
OPBCZTE3    ....C.............A......G.....................G.T.....................G.....AG.
OPBCZTE4    ....C.............A......G.....................G.T........G..........G......G.
OPBCZYJ1    ....C.............A......G.....................G.T.....................G......G.
OPBCZCT1    ....C.............A......G.....................G.T.....................G......G.
OPBCZCT2    ....C.............A......G.......A.............G.T.....................G......G.
OPBCZCT3    ....C.............A......G.......A.............G.T.....................G......G.
OPBCZCT4    ....C.............A......G.....................G.T.....................G......G.
OPSCGPJ1    ...................A...........G......A......T..G..G.T.....................G...A..G.
OPSCGPJ2    ...................A.....................A.....G.T.....................G......GT
OPHCHWC1    AT..C.....A.CG.......A..C.....G......A......T....AG.T..T.......GA...C.-AG...A.A..
OPHCHND1    AT..C.....A.CG.......A..C.....G......A......T....AG.T..T.....G.GA...C.AAGC.CAGA.T
OPHCHND2    AT..C.....A.CG.......A..C.....G......A......T....AG.T..T.......GA...C.AAGC.CAGA.T
OPHCHND3    AT..C.....A.CG.......A..C.....G......A......T....AG.T..T.......GA...C.AAGC.CAGA.T
OPHCHLS1    AT.CC.....A.CG.......A..C.....G......A......-....AG.T..T.......GA...C.-AG.G.A.A.T
OPHCHLS2    AT.CC.....A.CG.......A..C.....G......A......-....AG.T..T.......GA...C.-AG...A.A.T
OPHCHTT1    AT..C.....A.CG.......A..C.....G......A.....TT....AG.T..T.....G.GA...C.-AG...A.A.T
OPHCHTT2    AT..C.....A.CG.......A..C.....G......A.....TT....AG.T..T.......GA...C.-AG...A.A.T
OPHCHTT3    AT..C.....A.CG.......A..C.....G......A.....TT....AG.T..T.....G.GA...C.-AG...A.A.T
OPHCHCH1    AT..C.....A.CG.......A..C.....G......A.....-T....AG.T..T.......GA...C.AAG...A.A.T
PASCGPJ1    AC-.CAACTTATCGTTTAAGT.CA.GGGAA.AT.TCCTGCCAC..AT..T.A.TA.CTCTA.T.AAAGCGC.-...A.C.-
PASCGPJ2    AC-.CAACTTATCGTTTAAGT.CA.GGGAA.AT.TCCTGCCAC..AT..T.A.TA.CTCTA.T.AACGCGC.-...A.C.-
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