

TAXONOMIC REVISION AND MITOCHONDRIAL SEQUENCE EVOLUTION OF THE CYPRINID GENUS *SQUALIDUS* (TELEOSTEI: CYPRINIDAE) IN TAIWAN WITH DESCRIPTION OF A NEW SPECIES

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ABSTRACT. – Taxonomic studies of the cyprinid genus *Squalidus* was carried out in Taiwanese waters and three distinct species could be recognized. Among them, the endemic species, *S. ijimae* (Oshima, 1919) is only distributed around two river basins of Northwestern Taiwan. The slender species, *S. argentatus* (Sauvage & Dabry de Thiersant, 1874) is a newly-recorded species in Taiwanese waters and is only found in the Tanshuei River basin. A new species, *S. banarescui* has been discovered recently in the freshwater Wu River basin in Central Taiwan. The molecular phylogenetic studies also revealed that the three species are genetically distinct and there is a closer relationship between *S. ijimae* and *S. banarescui* than between *S. ijimae* and *S. argentatus*. The analysis of mitochondrial DNA (mtDNA) phylogeny shows the monophyletic group for the three Taiwanese species.

KEY WORDS. – *Squalidus*, new cyprinid, molecular evolution, primary freshwater fish, Taiwan.

INTRODUCTION

The genus, *Squalidus* (Dybowsky, 1872), is a distinct small-sized cyprinid genus with a translucent and slender body. It always has a pair of barbels and two rows of pharyngeal teeth. The genus is widely distributed in the lower or middle reaches of Eastern Asia from Korea, Japan, mainland China to Taiwan (Banarescu, 1961, 1962; Banarescu & Nalbant, 1964; Hosoya & Jeon, 1984; Chen, 1998; Chen & Fang, 1999; Nakabo, 2000; Kawanabe et al., 2002). There are at least seven valid species of *Squalidus* from mainland China and one endemic species reported from Taiwan (Chen, 1998).

The exact number of species of *Squalidus* naturalized in the Taiwan islands from mainland China is not known. Oshima (1919) mentioned a single species under the genus *Gnathopogon* in Taiwan which had been replaced into *Squalidus* by Banarescu (1969; 1973). However, no further intensive survey was done on this genus. More recently, at least 230 species of inland water fishes in Taiwan have been reported (Chen & Fang, 1999; Chen & Chang, 2005) and this has documented the validity of *Squalidus* in Taiwan. We found three distinct morphological morphs which will also be tested by molecular sequence evidence. The collection can be represented by two major groups: the elongate form (one

morph with a distinct colour pattern) and the robust form (two morphs with distinct colour patterns). The meristic approach on the robust form failed to distinguish the two morphs, but employing mitochondrial DNA (mtDNA) sequences (Chen et al., 1998, 2002) provided evidence for the three morphs with distinct colour patterns to be recognized as three valid species. Herein, we summarize and describe a new sibling *Squalidus* species from Taiwan with comparative mitochondrial evolutionary data as well as providing an artificial key to all nominal species of *Squalidus* in Taiwan.

MATERIALS AND METHODS

All cyprinid fish specimens of genus *Squalidus* were collected by either cast-netting or angling. Specimens used for morphological studies were preserved in 10% formalin before being transferred into 70% ethanol for long-term preservation. The meristic count and morphometric measurements generally follow the methods of Hosoya & Jeon (1984). All Taiwanese specimens of the genus *Squalidus* collected within the last 6 years were deposited in the Pisces collection of National Taiwan Ocean University (NTOU P), Keelung, Taiwan. The classical types for American collection were deposited in the Field Museum of Natural History (FMNH),

Chicago, USA. The French collection is in the Muséum national d'Histoire naturelle (MNHN), Paris, France. Meristic abbreviations are as follows: A = anal fin rays; D = dorsal fin rays; L.L = lateral-line scales; P = pectoral fin rays; Ph = pharyngeal teeth; PreD = predorsal scale rows; TR1 = transverse scale rows above lateral-line scales and V = ventral fin rays.

The fresh specimens of *Squalidus* used for molecular analysis were directly preserved in 95% ethanol when caught and transferred frozen after preservation to the laboratory. In this study, five cyprinid species were used in the molecular analysis and phylogenetic studies. They are the three morphological forms of *Squalidus* from Taiwan, *Squalidus atromaculatus* (Nichols & Pope, 1927) from Guangshi Province, China and *Candidia barbata* (Regan, 1908b) that was assigned as an outgroup.

All DNA extraction of the cyprinids was done according to the general protocols of the phenol-chloroform method (Sambrook et al., 1989; Chen et al., 1998, 2002). The DNA fragments of about 1,500 bp, including the full length of D-loop region, were amplified by polymerase chain reaction (PCR) using primers based on the flanking region that were designed from the sequences of tRNA-PHE and 12S rRNA (Chen et al., 1998, 2002). PCR was done in a Model 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 (5 µM), 0.1 µL of 0.5 unit Ex *Taq* (Takara) and 1.0 µL of template. The thermal cycle 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 DNA 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 (Boehringer Mannheim, High Pure PCR Product Purification kit), before undergoing direct cycle sequencing with dye-labeled terminators (ABI Big-Dye kit). The primers used were the same as those for PCR. All sequencing reactions were performed according to the manufacturers' instructions. Labeled fragments were analyzed using an ABI Model 377-64 DNA sequencer (ABI). Nucleotide sequence alignment was done manually. The phylogenetic and molecular evolutionary analyses were conducted using Molecular Evolutionary Genetics Analysis (MEGA) version 3.0 (Kumar et al., 2004) by neighbor-joining (NJ) and maximum parsimony (MP) methods with 1,000 bootstrap replications (Fig. 4).

TAXONOMY

Squalidus Dybowski, 1872

Type species. – *Squalidus chankaensis* (Dybowski, 1872)

Squalidus argentatus (Sauvage & Dabry de Thiersant, 1874) (Fig. 1)

Gobio argentatus Sauvage & Dabry de Thiersant, 1874: 9.
Gobio (Leucogobio) hsui Wu & Wang, 1931: 227.
Gobio (Squalidus) argentatus Banareescu, 1961: 321.
Gnathopogon iijimae Tseng, 1986: 62 (not Oshima, 1919).
Squalidus chankaensis argentatus Banareescu & Nalbant, 1973: 89.
Squalidus argentatus Yue in Chen, 1998: 314.

Materials examined. – Holotype - MNHN 5038, 98.9 mm SL, Yangtsi River, China.

Others. – MNHN 5039, 98.1 mm SL, locality data same as holotype above; NTOU P-2005-07-014, 2 specimens, 70.6 - 74.2 mm SL, Ji-Tan, Shin-Tein-Shi, Tanshuei River basin, Taipei County, Taiwan, coll. I-S. Chen, Aug. 1999; NTOU P-2005-07-015, 3 specimens, 72.3 - 78.0 mm SL, Shin-Tien-Shi, Tanshuei River basin, Taipei County, Taiwan, coll. Y.C. Chang, Jul. 2004.

Diagnosis. – This species is characterized from congeneric species by the unique combination of the following features: dorsal fin rays 3 + 7, anal fin rays 3 + 6, pectoral fin rays 1 + 14; lateral-line scales 39 - 41 (modally 40), predorsal scales 12 - 13; body rather slender, the transverse rows of papillae on snout loosely arranged and very long barbels over 2/3 of eye diameter and body with longitudinal grayish and golden stripes; dorsal side scattered with few irregular black spots; a longitudinal row of grayish black blotches along lateral body; all fins unmarked.

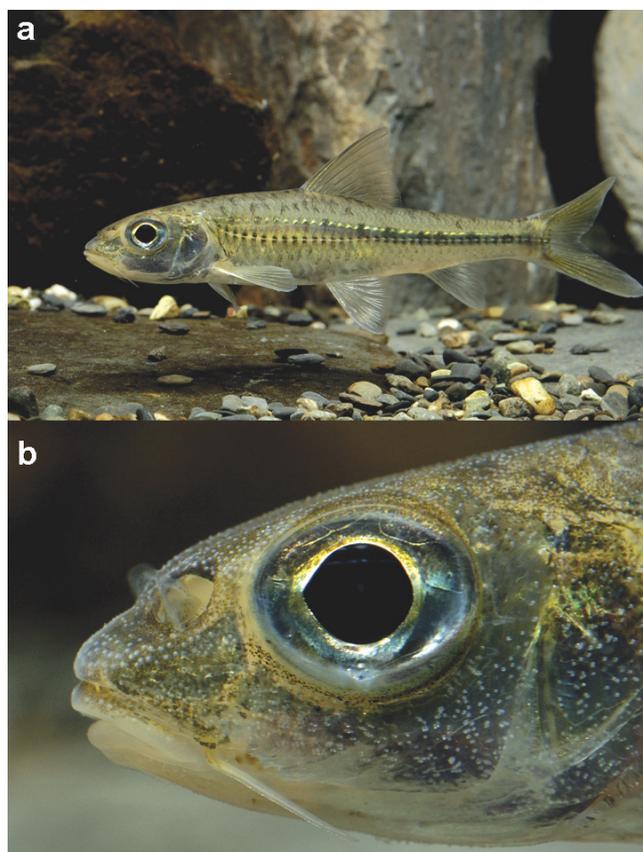


Fig. 1. *Squalidus argentatus*, Tanshuei River, Taipei County, Taiwan. a) lateral view, b) close-up of head, lateral view.

Distribution. – This species is the first record in Taiwanese waters and is only found in Shin-Tein-Shi, the middle reaches of Tanshuei River basin in Northern Taiwan.

Remarks. – This species is most likely to be *Squalidus argentatus*. However, there is a need to survey the Chinese waters to determine this species complex, especially in the Yangtsi River basin as well as all other remaining basins South of the Yangtsi in mainland China.

Squalidus ijimae (Oshima, 1919)
(Fig. 2)

Gnathopogon ijimae Oshima, 1919: 219.

Squalidus ijimae Banareescu & Nalbant, 1973: 97. (in part, type specimen only).

Squalidus ijimae Chen & Fang, 1999: 92.

Materials examined. – Holotype - FMNH 59096, 63.0 mm SL, Tozen River (Shin-Chu County), Taiwan, coll. T. Aoki, Dec.1916.

Others. – NTOU P-2005-07-016, 6 specimens, 48.2 - 67.3 mm SL, Holong River, Miao-Li County, Taiwan, coll. Y.C. Chang, Jul.1999.

Diagnosis. – *Squalidus ijimae* (Oshima, 1919) is characterized from congeneric species by the unique combination of following features: dorsal fin rays 3 + 7, anal fin rays 3 + 6, pectoral fin rays 1 + 13 - 14 (modally 13); lateral-line scales 34 - 35 (modally 35), predorsal scale rows

10 - 11; body robust, the transverse rows of papillae on snout loosely arranged and maxillary barbels short and approximately equal to 1/2 of eye diameter and body greenish-silver on dorsal and silver-white on ventral side; body scale pocket, with grayish posterior margin, has net-like patterns; body with a longitudinal grayish stripe; all fins translucent; dorsal and caudal fin with minute black spots; other odd fins unmarked.

Distribution. – This endemic species is found only in the type locality, the Tozen River in Shin-Chu County (from Oshima's original description and type) and the nearby Holong River in Miao-Li County (from our collection) in Western Taiwan. However, we were not able to locate this species for the present study from the Tozen River. This was despite more intensive investigating and surveying of inland water fishes during the past 15 years.

Remarks. – Although the single type specimen is poorly preserved and dehydrated, the meristic features, like body scales and fin rays, clearly match the current specimens found in the Holong River basin. The transverse rows of papillae on snout are also loosely arranged which is specific to the specimens found in the current species of both basins.

Squalidus banareescui, new species
(Fig. 3)

Gnathopogon ijimae Oshima, 1920: 128 (not Oshima, 1919).

Squalidus ijimae Banareescu, 1969: 100 (not Oshima, 1919).

Squalidus ijimae Banareescu & Nalbant, 1973: 97 (not Oshima, 1919) (in part, type specimen excluded).

Materials examined. – Holotype - NTOU P-2005-07-017, 60.5 mm SL, male, Wu (Ta-du) River, Taichung County, Taiwan, coll. Y.C. Chang, Jul.1999.

Paratypes. – NTOU P-2005-07-018, 11 specimens, 36.8-63.0 mm SL, Wu (Ta-du) River, Taichung County, Taiwan, coll. Y.C. Chang, Jul.1999.

Diagnosis. – This new species is characterized from congeneric species by the unique combination of following features: dorsal fin rays 3 + 7 - 8, anal fin rays 3 + 6, pectoral fin rays 1 + 13; lateral-line scales 34 - 36 (modally 35), predorsal scales rows 10 - 11 (modally 11); body robust, the transverse rows of papillae on snout densely arranged and short barbels approximately equal to 1/2 of eye diameter and body greenish-silver on dorsal and silver-white on ventral side; dorsal side scattered with some irregularly black spots; snout with a dark bar on lateral side; belly unmarked or having a few dark spots on lateral side, a row of 6 - 7 grayish-black longitudinal blotches along lateral side of body; each lateral-line scale with an L-shaped black mark; dorsal and caudal fin with few rows of minute black spots; body with longitudinal grayish and silver stripes.

Description. – D. 3 + 7 - 8 (modally 7); A. 3 + 6; P. 1 + 13; L.L. 34 - 36 (modally 35); TR1. 5 - 6 (modally 5); V. 1 + 7; Ph. 5.3 - 3.5 (frequency distribution listed in Table 1). Body

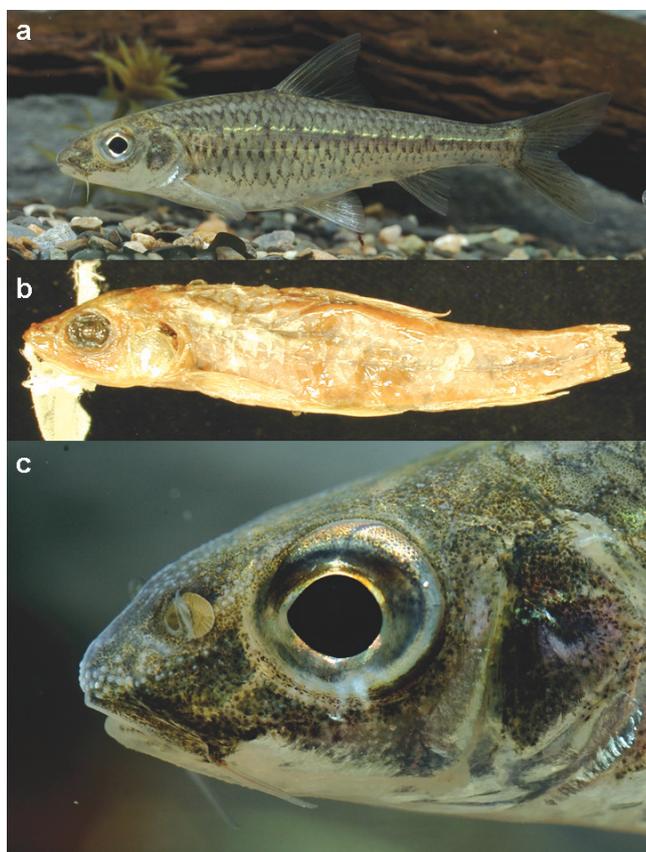


Fig. 2. *Squalidus ijimae*. a) lateral view, b) holotype, FMNH59086, 63.0 mm SL, Tozen River, Shin-Chu County, lateral view, c) close-up of head, Holong River, Miao-Li County, Taiwan, lateral view.

elongated, robust and compressed. Body scales medium-sized. Lateral-line scales complete, extending slightly downward ventrally to caudal fin base along the mid-lateral. Head moderately large. Mouth terminal, slightly oblique, almost extending to anterior margin of orbit. Snout straight in dorsal profile and the tip pointed. The transverse rows of papillae on dorsal snout densely arranged. A pair of barbels on corner of mouth and the length is approximately 1/2 of eye diameter. Upper jaw more prominent. Eye very large and high on the head. Cheek low. Very tiny tubercles on snout in male. Inter-orbital region wide. Dorsal fin origin is closer to snout tip than to caudal fin base. Anal fin base short. Pectoral fin moderate in size, not extending to ventral fin origin. All morphometric data are listed in Table 2.

Coloration. – Body greenish-silver on dorsal and silver-white on ventral side. Dorsal side scattered with some irregular black spots. Snout with a deep grayish-black bar on lateral side which extends to lower lip and chin. Ventral half of body unmarked or with just a few spots. A row of 6 - 7 grayish-black longitudinal blotches along lateral body. Each lateral-line scale with an L-shaped black mark on anterior part. All fins translucent and grayish in adult. Dorsal and caudal fins with a few rows of minute black spots. Body with longitudinal grayish and silver stripes.

Distribution. – This new species is only found in the middle and lower reaches of the Wu (Ta-du) River basin in Central Taiwan. There is a high possibility that it is an endemic to Taiwan.

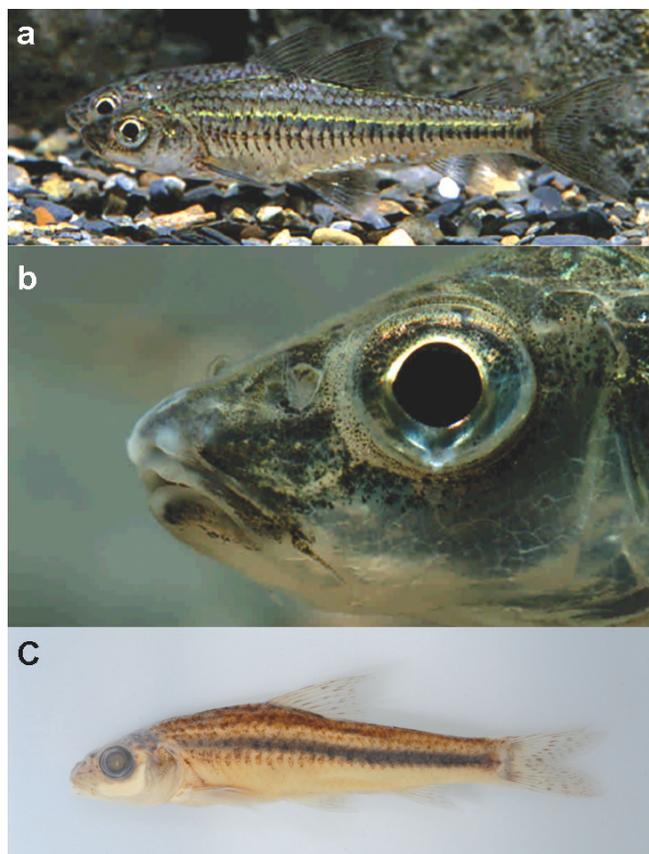


Fig. 3. *Squalidus banarescui*. a) lateral view, b) close-up of head, lateral view, c) holotype, NTOU P- 2005-07-017, 60.5 mm SL, Wu River, Taichung County, Taiwan, lateral view.

Etymology. – The specific name, *banarescui*, is named after Prof. P. Banarescu, in recognition to his great academic contribution to the Taiwanese cyprinid taxonomy, especially on the subfamily, Gobioninae between 1960 and 1973.

Comparison of related species. – *Squalidus banarescui* shares a robust body shape as well as low counts of lateral-line scales with the following species from mainland China: a) *S. wolterstorffi* (Regan, 1908a); b) *S. nitens* (Günther, 1873) and c) *S. intermedius* (Nichols, 1929). *S. banarescui* is separated from *S. wolterstorffi* and *S. intermedius* by dark spotted dorsal and caudal fins (vs. unmarked dorsal and caudal fins). Although *S. nitens* also has spotted caudal and dorsal fins, it can be distinguished from *S. banarescui* by the long maxillary barbels (about half of the orbit), while the maxillary barbels in *S. nitens* are short (much less than half of the orbit). *S. banarescui* and *S. nitens* can be further distinguished by their specific body colour patterns.

In Taiwan, *S. banarescui* seems to be most similar to *S. ijimai* in terms of body shape and overall meristic features, as compared to the other congeneric and endemic Chinese species. These two species are easily confused as they have very similar scale counts. Oshima (1920) and Banarescu (1969, 1973) concluded that they were the same species even when they were collected from distinct geographical regions because they only had access to a very poor holotype of *S. ijimai* without further fresh material. All of Banarescu's specimens were from the Wu River basin in Central Taiwan which was not a type locality of *S. ijimai* in Northwest Taiwan.

From our intensive collection for both species, they can be well separated by both morphological and molecular evidence. *Squalidus banarescui* is distinguished from *S. ijimai* by the following characters: a) snout pointed with straight dorsal profile in *S. banarescui* (vs. rounded with convex dorsal profile in *S. ijimai*); b) the transverse papillary rows on dorsal snout highly densely-arranged in *S. banarescui* (vs. loosely arranged in *S. ijimai*); c) scale pocket indistinct in *S. banarescui* (vs. with conspicuous grayish-black margin in *S. ijimai*); d) belly unmarked or with a few black spots in *S. banarescui* (vs. net-like dark pattern in *S. ijimai*); e) each lateral-line scale has L-shaped black marks in *S. banarescui* (vs. no marking in *S. ijimai*) and f) the confirmation of mitogenetic differentiation presented in the following section:

The complete mtDNA D-loop sequences of *Squalidus* have been sequenced from the three morphs (Table 3). The results clearly support the distinction of the three groups of haplotypes. However, the same haplotype was found within the species of all individuals that have been sequenced (ranging from 2 - 8 specimens for each species). Such genetic simplicity implies low mitogenetic diversity within the limited populations of Taiwanese *Squalidus* species inhabiting the lower and middle reaches. This is in contrast to the other Taiwanese cyprinid, the *Candidia* species complex with rather high haplotype diversity in the upper reaches of hill streams (Wu et al., 2007). The molecular phylogenetic studies have been analyzed and show the three Taiwanese species to be a

Table 1. Meristic features of the three species of the *Squalidus* from Taiwan.

Species	D		A			P			V				LL						
	3 + 7	3 + 8	Av	3 + 6	Av	1 + 13	1 + 14	Av	1 + 7	Av	34	35	36	37	38	39	40	41	Av
<i>S. argentatus</i>	5	-	7.0	5	6.0	-	5	14.0	5	7.0	-	-	-	-	-	2	6	2	40.0
<i>S. ijimae</i>	6	-	7.0	6	6.0	5	1	13.2	6	7.0	4	8	-	-	-	-	-	-	34.7
<i>S. banarescui</i> new species	10	2	7.2	12	6.0	12	-	13.0	12	7.0	3	16	5	-	-	-	-	-	35.1

Species	TRI			PreD				
	5	6	Av	10	11	12	13	Av
<i>S. argentatus</i>	5	-	5.0	-	-	1	4	12.8
<i>S. ijimae</i>	5	-	5.0	4	2	-	-	10.3
<i>S. banarescui</i> new species	10	2	5.2	4	8	-	-	10.7

Table 2. Morphometry of the three valid species of *Squalidus* from Western Taiwan.

Species	<i>Squalidus argentatus</i>			<i>Squalidus tijmae</i>		<i>Squalidus banarescui</i> new species	
	Holotype	Holotype + non-types	Non-types	Holotype	Holotype + paratypes		
Types of specimens	1	3	6	1	8		
No. of specimens	99.0	68.6 - 99.0	57.6 - 61.2	60.5	35.8 - 60.5		
Standard length, SL (mm)	25.0	25.0 - 27.1 (26.2)	26.8 - 30.1 (28.7)	28.6	26.2 - 29.1 (28.0)		
Head length, HL (% of SL)	22.6	19.9 - 22.6 (21.1)	21.4 - 24.1 (22.8)	23.6	21.6 - 25.7 (23.3)		
Body depth	NM	14.2 - 14.3 (14.2)*	12.8 - 15.6 (14.3)	12.3	12.3 - 16.3 (13.6)		
Body width	9.3	8.4 - 9.3 (8.9)	9.0 - 9.6 (9.3)	10.8	10.5 - 11.7 (11.0)		
Depth of caudal peduncle	16.3	16.3 - 19.4 (17.9)	16.7 - 18.9 (17.6)	15.3	14.8 - 16.7 (15.4)		
Length of caudal peduncle	46.4	46.4 - 48.2 (47.4)	47.1 - 50.3 (48.7)	48.0	47.5 - 50.2 (48.7)		
Predorsal length	76.0	74.7 - 76.0 (75.2)	73.1 - 76.4 (75.2)	73.1	72.0 - 73.7 (72.9)		
Prealan-fin length	48.4	48.4 - 51.0 (49.8)	51.9 - 56.8 (54.1)	47.9	46.2 - 49.2 (48.0)		
Preventral length	NM	54.0 - 55.2 (54.6)*	51.8 - 54.9 (53.6)	55.3	52.3 - 55.3 (54.2)		
Dorsal origin to caudal base	NM	25.3 - 26.6 (26.0)*	24.6 - 27.2 (25.5)	21.9	19.2 - 23.6 (21.9)		
Pectoral origin to pelvic insertion	NM	24.2 - 24.5 (24.5)*	22.3 - 25.6 (24.2)	26.2	23.8 - 28.0 (25.9)		
Length of longest dorsal soft ray	NM	17.1 - 17.7 (17.4)*	15.2 - 17.3 (16.7)	18.6	16.8 - 19.7 (18.5)		
Length of longest anal soft ray	NM	19.9 - 21.6 (20.7)*	18.0 - 18.9 (18.4)	20.4	17.5 - 20.4 (19.5)		
Length of longest pectoral soft ray	16.2	15.5 - 16.2 (15.8)	13.5 - 15.7 (15.0)	16.0	13.6 - 17.1 (15.7)		
Length of dorsal fin base	9.9	9.1 - 9.9 (9.6)	9.1 - 15.7 (10.7)	8.9	8.5 - 9.8 (8.9)		
Length of anal fin base	36.9	35.3 - 41.9 (38.0)	34.5 - 39.9 (36.4)	28.3	26.6 - 31.8 (29.4)		
Snout length (% of HL)	29.2	28.3 - 32.4 (30.0)	27.7 - 32.9 (30.6)	43.1	37.6 - 47.7 (44.1)		
Interorbital width	32.6	32.6 - 35.7 (34.2)	29.1 - 33.2 (31.0)	29.2	27.2 - 32.1 (29.0)		
Eye diameter	29.9	27.9 - 29.9 (29.2)	18.3 - 22.7 (20.8)	13.3	13.3 - 18.6 (15.1)		
Barbel length							

NM = not measured; * = data was from two specimens instead of the holotype as there was no data available for some features.

very grateful to Dr. H. H. Ng for helping to check the rather detailed information of the holotype of *Squalidus ijimae* from FMNH. ISC also extends his thanks to Dr. B. Seret for his hospitality while examining the type specimens in the Paris Museum in 1999.

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