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Molecular Identification of Two Sibling Species of *Puntius* in Taiwan

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Chia-Hao Chang, Yi-Ta Shao, and Hsiao-Wei Kao (2006) Molecular identification of two sibling species of *Puntius* in Taiwan. *Zoological Studies* **45**(2): 149-156. *Puntius* fish from Taiwan and South China were collected and analyzed. Specimens from the northern and central Taiwan were characterized by the absence of barbels. On the contrary, specimens from the southern Taiwan and South China were characterized by the presence of barbels. These characteristics morphologically match *P. snyderi* and *P. semifasciolatus* respectively (Oshima 1919). We hypothesized that there were two species of *Puntius* in Taiwan. To test this hypothesis, we amplified and sequenced the cytochrome b gene from fish specimens of China and Taiwan. Phylogenetic analysis revealed the existence of two major clades of *Puntius* fishes with an average genetic distance of 0.12 between them. The fish specimens from southern Taiwan were clustered with *P. semifasciolatus* of China, but fish specimens from northern and central Taiwan were clustered together. The estimated evolutionary rate of cytochrome b gene for *Puntius* was 0.368% per million yrs (MY). Thus the divergence time between *P. snyderi* and *P. semifasciolatus* was about 26.93 million years ago (MYA) and the divergent time between Taiwan's and China's *P. semifasciolatus* was about 4.40 MYA. Taken together, our results supported the existence of two species of *Puntius* fishes in Taiwan. *P. snyderi* is distributed in northern and central Taiwan, while *P. semifasciolatus* is in southern Taiwan. http://zoolstud.sinica.edu.tw/Journals/45.2/149.pdf

Key words: Cytochrome b gene, Freshwater fish, Molecular clock, Phylogenetic tree.

Puntius snyderi is a freshwater cyprinid fish discovered by Oshima when he collected the freshwater fishes in Taiwan in 1915-1917. It was mainly distributed in northern and central Taiwan (Oshima 1919) in contrast to another species, *P. semifasciolatus* that was distributed in Pingtung, southern Taiwan (Oshima 1919, Chen 1969). The major morphological difference between these two fishes is the presence of a pair of maxillary barbels in *P. semifasciolatusi* but their absence in *P. snyderi*. Because of similarities of their morphology and distribution, the absence of official collecting records of *P. snyderi* since 1927, and the disappearance of the holotype of *P. snyderi*, *P. snyderi* was regarded as a synonym of *P. semifasciolatus* by (Shen 1984). The issue of species validity was also reflected in the books of (Tzeng 1986), (Shen et al. 1993), and (Chen and Feng 1999) which only described *P. semifasciolatus* in Taiwan. In their books, its distribution ranged from the northern and central parts of Taiwan to the southern part of western Taiwan. Obviously, this geographic distribution was a combination of the areas where *P. snyderi* and *P. semifasciolatus* were once respectively reported.

During collections of freshwater fish of Taiwan and China, we noticed the existence of two major forms of *Puntius* fish in Taiwan. Fish from central Taiwan were characterized by the absence of barbels and a smaller number of horizontal black bands (crossbars), but fish from the southern Taiwan and China had one pair of barbels and had a greater number of and thinner crossbars (Fig. 1). Because these characteristics respectively matched those reported for *P. semifasciolatus* and *P. snyderi*, we hypothesized that there were two

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species of *Puntius* in Taiwan.

Mitochondrial DNA sequences are frequently utilized for inferring phylogenetic relationships among organisms, because they have the properties of large copy number, faster evolutionary rate, maternal inheritance, smaller molecular weight, and a lack of introns (Brown et al. 1979, Moritz et al. 1987). Among genes, the mitochondrial cytochrome b has been widely used in various vertebrates as a genetic marker for species-level identification (Johns and Avise 1998). For this reason, we also chose cytochrome b as a genetic marker to resolve the relationships among the fish specimens we collected. We collected *Puntius* fishes from Taiwan and China. Morphological characters of specimens either collected by us or deposited in museums of Taiwan were examined. Phylogenetic trees were constructed using the cytochrome b gene. In addition, cytochrome b sequences of *Barbus*, *Capoeta*, *Linichthys*, and *Puntius* were also downloaded from GenBank and analyzed because *P. semifasciolatus* had been placed in all of these genera at some point in the past (Günther 1868, Oshima 1919, Herre and Myers 1931). We finally estimated the divergence time between and among the specimens from Taiwan and China in order to trace the speciation event.

MATERIALS AND METHODS

Sampling of specimens

Because of habitat destruction or water pollution, *Puntius* specimens were only collected at four locations in Taiwan (Fig. 2), and one location in China (Fig. 2) in this study, although extensive efforts were also made on the island of Kinmen (Fig. 2). We collected the *P. snyderi* at Sanyi in

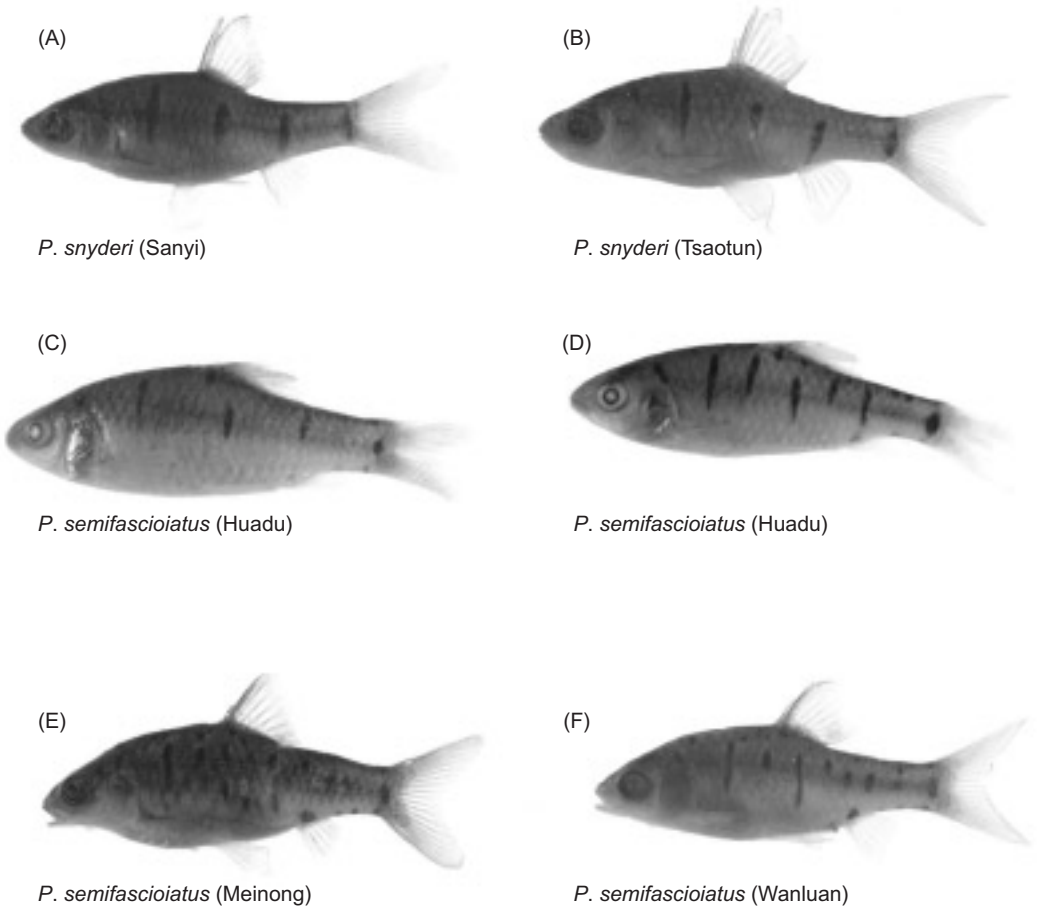


Fig. 1. Crossbar pattern of *Puntius semifasciolatus* and *P. snyderi* from different collection locales. (A) *Puntius snyderi* in Sanyi, Taiwan; (B) *P. snyderi* in Tsaotun, Taiwan; (C) *P. semifasciolatus* in Huadu, China; (D) *P. semifasciolatus* in Huadu, China; (E) *P. semifasciolatus* in Meinong, Taiwan; (F) *P. semifasciolatus* in Wanluan, Taiwan.

northern Taiwan and Tsaotun in central Taiwan, and *P. semifasciolatus* in Meinong and Wanluan in southern Taiwan. Southern, northern, and central Taiwan were distinguished according to (Chen and Feng 1999). Fish specimens were also collected in Huadu, Guangdong in South China. Fish specimens were either collected by traps or fishing. For DNA extraction, a piece of muscle tissue was excised and placed in 95% alcohol for DNA extraction. The remaining part of the specimen was fixed in 30% formalin, then stored in the 70% alcohol for morphometric measurements. Specimens of *P. semifasciolatus* from Xiulu and Cenjia in Hainan (NTUM01985, NTUM01988) and Penghu (NTUM00429) (Fig. 2) deposited in the Department of Life science, National Taiwan University were also examined.

Crude DNA extraction

Specimens for each sampling sites were chosen for DNA extraction. Crude DNA extraction was followed the Gentra-DNA extraction protocol (Gentra system, Minneapolis, MN55441, USA).

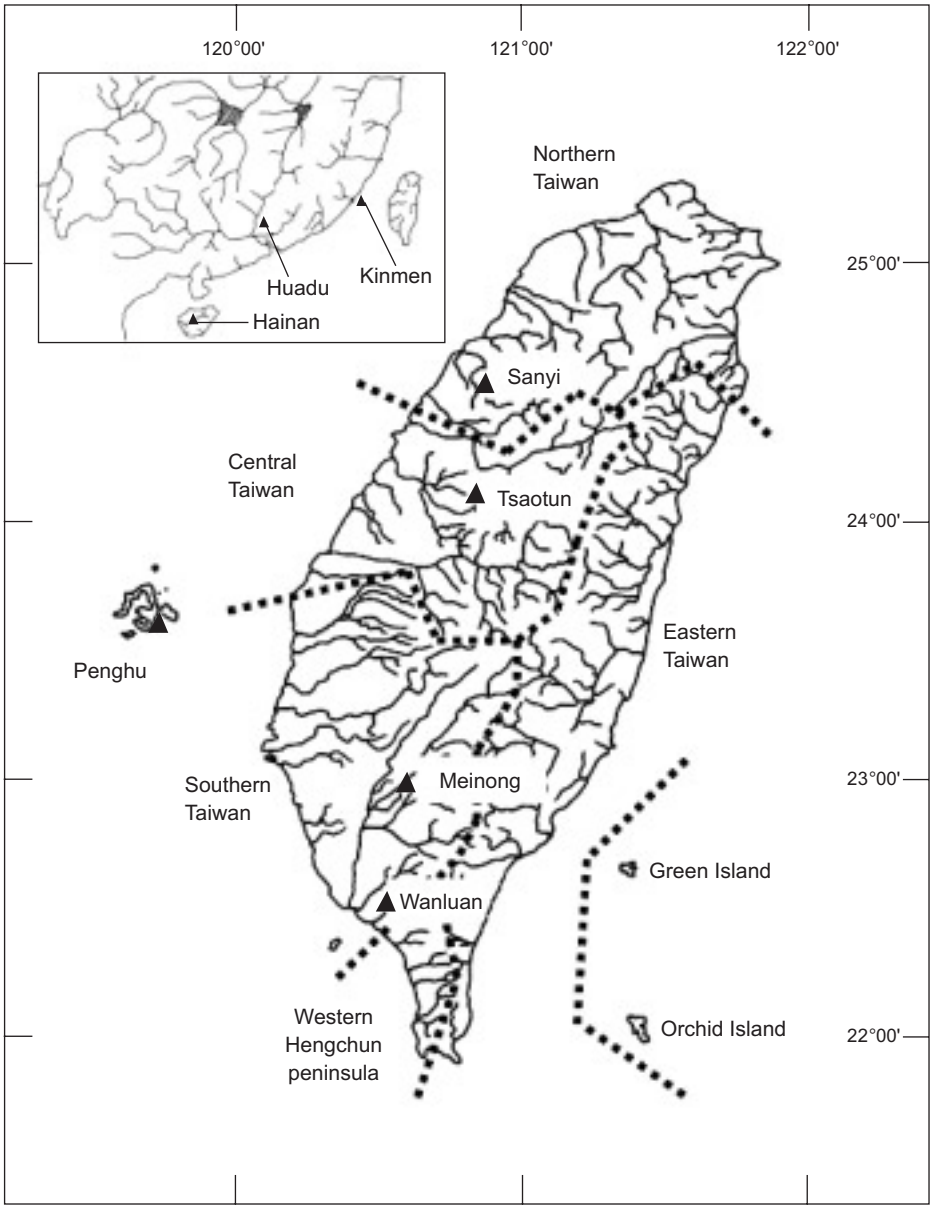


Fig. 2. Locations of sampling sites of *Puntius snyderi* and *P. semifasciolatus*, and the zoogeographical distribution regions of freshwater fishes in Taiwan.

Crude DNA samples were stored at -20°C. A fragment of 1140 bp of the mitochondrial cytochrome b gene was amplified by polymerase chain reaction (Applied Biosystems 2700, USA). Briefly, each 100μl PCR reaction contain about 10 ng template DNA, 10μl 10x reaction buffer, 8μl dNTP mix (2.5 mM dNTP each), 25μmol of each specific primer, Cyto1 (5'-GTTATTCAACTACAA-GAACTAC-3') and Cyto2 (5'-TTTAGAC-TAAGCTACTAGGGCA-3'), 2.5 U of *Taq* polymerase (TaKaRa Taq®, Otsu, Shiga, 520-2193, Japan), and distilled water. Thermal cycling began with a single denaturation step at 94°C for 4 min, then 35 cycles were performed consisting of denaturation at 94°C for 1 min, annealing at 55-65 °C for 1 min, and extension at 72°C for 1 min. Finally, a single extension step at 72°C for 10 min was utilized to complete the extension of DNA fragments. PCR products were purified with PCR DNA Fragments Extraction Kit (Geneaid DF100, Taipei, Taiwan). Approximately 50 ng of double-strand PCR product was used in the cycle sequencing reactions with the same primers following the protocol of ABI PRISM BigDye sequencing Kit (PE Biosystems, Foster City, CA94404, USA). Reaction products were electrophoresed on an ABI model 3100 version 3.7 automated sequencer (Applied Biosystems). Contiguous sequences from two directions of the cytochrome b gene were created using the program BioEdit ver. 5.0.9. In total 20 sequences of the *P. semifasciolatus* and *P. snyderi* in this study were submitted to GenBank under the accession numbers AY856105-AY856116 and AY85697-AY856104. For calibrating the evolutionary rate and constructing the phylogenetic tree, sequences of *Capoeta capoeta* (AF145951), *Capoeta c. angorae* (AF145950), *C. trutta* (AF145949), *P. titteya* (AF287455), *P. conchonius* (AY004751), *P. semifasciolatus* from China (AF309505), *Barbus barbus* (Y10450), *Linichthys laticeps* (AF854739), *Polypterus ornatipinnis* (NC001778), *Elops hawaiensis* (AB051070), *Osteoglossum ferreirai* (AB035239), *Cyprinus carpio* (NC001606), and *Engraulis japonicus* (NC003097) were downloaded from GenBank.

Phylogenetic analysis and estimation of divergence times

We used two methods to construct the phylogenetic tree: First, we employed the computer program ModelTest vers. 3.06 (Posada and Crandall 1998) to determine the most-suitable maximum-likelihood (ML) model. The maximum likelihood tree was constructed by the PAUP* program (Swofford 2001) with the most-suitable model. Because *Puntius* is among the most-primitive group in the subfamily Barbina, it is inappropriate to choose species of the same genus or other genera in this subfamily as an outgroup. Although fossil record have revealed that the Cypininae branched out the earliest among the other three closely related subfamilies of the Barbinae, Schizothoracinae, and Labeoninae (Chen et al. 1998), this is yet unsupported by any phylogenetic analysis. For this reason, we constructed an unrooted tree. Statistical support of the tree was examined by bootstrapping 250 times. Second, we utilized the MrBayes program vers. 3.0 software (Ronquist and Huelsenbeck 2003) to conduct Bayesian inferences of phylogeny. In this analysis, sequences were analyzed using first, second, and third codons (Yang et al. 1998), respectively. Probabilistic inference using the method of Markov Chains of Monte Carlo (MCMC) was set to 2×10^6 times and burn-in was set to 10,000.

The evolutionary rate of the molecular clock was calibrated using Tammura-Nei distance. We used two methods to estimate the evolutionary rate of cytochrome b gene. First, fish fossil records were used that included *Polypterus ornatipinnis* (polyteriformes, 245 MYA), *Osteoglossum ferreirai* (osteoglossiformes, 208 MYA), *Elops hawaiensis* (elopiformes, 146 MYA), *Engraulis japonicus* (clupeiformes, 146 MYA), *Puntius semifasciolatus* (cypriniformes, 146 MYA), and *Cyprinus carpio* (cypriniformes, 23.5 MYA) (Jefferies 1995, Maisey 1996). We conducted the relative rate tests (Tajima's test; Tajima 1993) to ensure that evolutionary rate of the cytochrome b gene among these fishes exhibited no statistically significant differences. A species was excluded from the analysis if its rate significantly differed from those of the others. For this reason, the sturgeon (Acipenseriformes) and eel (Elopomorpha) were excluded from the calculation. An average evolutionary rate was used for calibration of divergence time using linearized tree program (Takezaki et al. 1995). Second, we assumed that no gene flow had occurred between the populations of *P. semifasciolatus* in Taiwan and China since the most-recent land bridge had been breached by rising seas about 15000 yrs ago (Emery et al 1971). The evolutionary rate was calculated by the genetic distance of *P. semifasciolatus* between Taiwan and China and the time of isolation (15000 yrs).

RESULTS

Morphological characters

Morphometric measurements are listed in table 1. Data for *P. snyderi* and *P. semifasciolatus* from (Oshima 1919) and (Chen et al. 1998) are also included for the purpose of comparison and identification of *Puntius* species in Taiwan. The major difference between *P. semifasciolatus* and *P. snyderi* was the presence or absence of barbels. *P. semifasciolatus* had 1 pair maxillary barbels but *P. snyderi* did not (Oshima 1919). Our specimens collected from Sanyi (Taiwan, Fig. 2) and Tsaotun (Taiwan, Fig. 2) matched *P. snyderi* (Oshima 1919), and specimens from Meinong (Taiwan, Fig. 2), Wanluan (Taiwan, Fig. 2) and Huadu (China, Fig. 2) matched the characters of *P. semifasciolatus*. For convenience of description, the species from northern and central Taiwan was regarded as *P. snyderi*, and the species from southern Taiwan and South China was regarded as *P. semifasciolatus* hereafter. The species validity of these two species was further tested by analyzing the cytochrome b gene.

Phylogenetic analysis and estimation of divergence times

The pairwise distances calculated by the K2P model are listed in table 2. The average pairwise distances between the specimens of *P. semifasciolatus* from South China and Taiwan were 0.027, and those between the specimens of *P. semifasciolatus* from South China and *P. snyderi* from Sanyi and Tsaotun of central Taiwan were 0.120 and 0.118 respectively. The best model selected by the program ModelTest was TVM+I+G with a gamma parameter of 1.0533 and the proportion of invariable sites was 0.5143.

The tree constructed by the Bayesian method had the same topology with that of the ML tree (Fig. 3). For evaluation of statistic support, both the posterior probabilities of the Bayesian method and bootstrap values of the ML tree are shown on the branches. There were four major clades supported by high values of posterior probabilities and bootstrap values in the tree. Clade I included *P. titteya* and *P. conchonius*; clade II included *B. barbus*, *C. trutta*, *C. capoeta*, and *C. capoeta angorae*; clade III included *P. snyderi* from northern and central Taiwan; and clade IV included *P. semifasciolatus* from China and southern Taiwan. Importantly, specimens of *P. semifasciolatus* from southern Taiwan were clustered with those of *P. semifasciolatus* from South China with very high statistical support. Specimens from northern and central Taiwan were clustered together (Fig. 3).

The evolutionary rate of cytochrome b gene estimated by the 1st method was 0.368 % per million years (MY) and 89.567% per MY by the 2nd method. It is evident that the 89.567% per MY estimated by the 2nd method is unreasonably high. Thus the estimated divergence time between *P. semifasciolatus* and *P. snyderi* was 26.93 MY and estimated divergence time of *P. semifasciolatus* between the specimens from South China and southern Taiwan was 4.40 MY.

DISCUSSION

Morphometric differences

Although the numbers of maxillary barbels and horizontal bars are used to distinguish four species of *Puntius* in China (Yue et al. 2000), the presence and absence of barbels is the only useful marker for distinguishing *P. snyderi* from *P. semifasciolatus*, because the number of the horizontal bars in *P. semifasciolatus* is variable among its different populations (Fig. 1).

We noted that numbers of dorsal and anal rays in both *P. synderi* and *P. semifasciolatus* are differed between our count and that of (Oshima 1919). The 1st spiny-soft rays of the dorsal and anal rays are very small and embedded under the skin which might account for the discrepancy between the measurements. This conclusion was drawn from two pieces of evidence. First, there are four spiny-soft dorsal rays and two spiny-soft anal rays in *P. semifasciolatus* documented in the book of the *Fauna Sinica Osteichthyes Cypriniformes II* (Chen 1998). Second, specimens of *Puntius* fishes in Hainan, Penghu, southern Taiwan, and Huadu we examined all had four spiny-soft rays in the dorsal fin and three spiny-soft rays in the anal fin regardless the species or locality we collected.

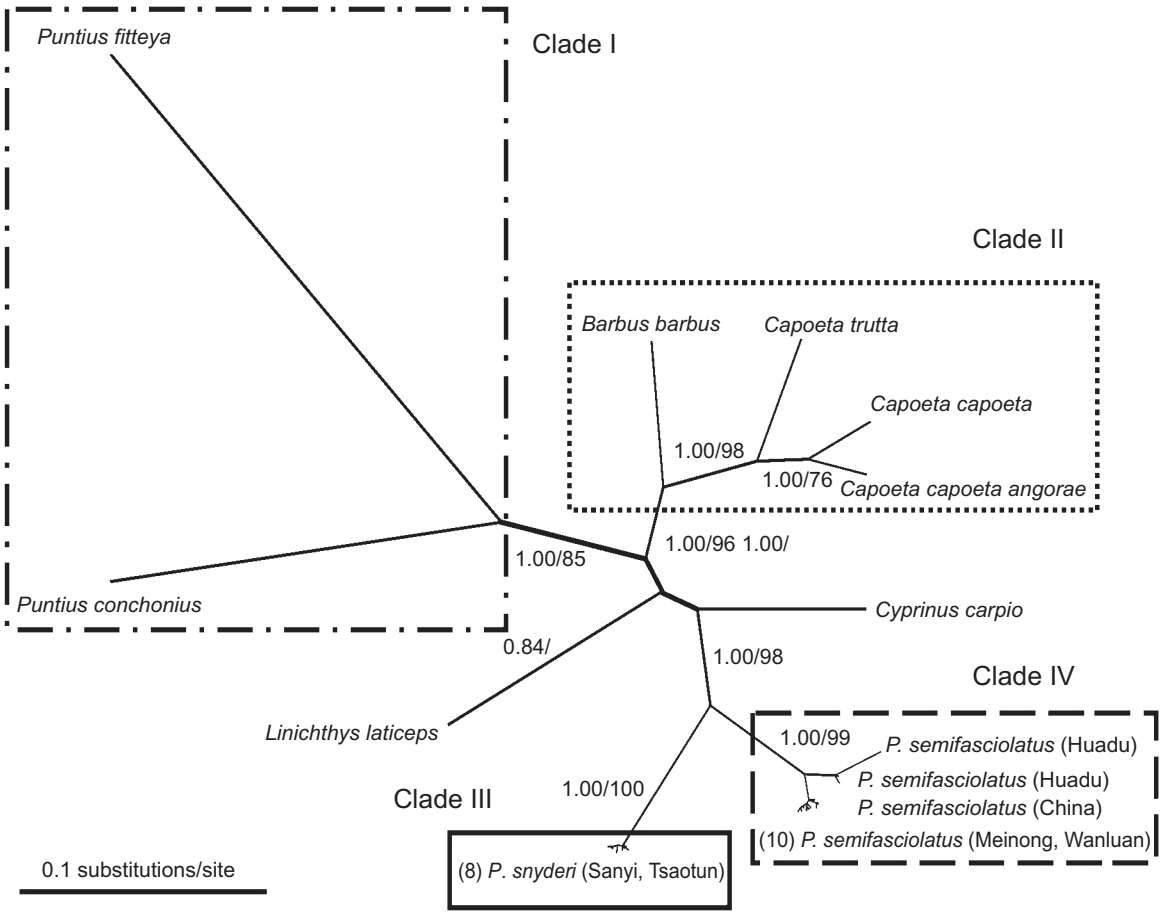
The horizontal bars of *P. semifasciolatus* from Huadu were very similar to those of *P. snyderi* because they both lacked minor bands (spots) on their bodies (Fig. 1) in contrast to those of *P. semifasciolatus* from Taiwan which had many minor bands and tiny spots on their bodies (Fig. 1). It is interesting that the crossbar pattern of the *P. semifasciolatus* from Huadu was similar to that of the *P. snyderi*. However, both kinds of crossbar patterns

were observed in the specimens of *P. semifasciolatus* from Hainan. Therefore *P. snyderi* once having been regarded as *P. semifasciolatus* might have been due to similarities of the crossbar pattern.

Genetic distance and phylogenetic tree

The genetic distance of the cytochrome b gene between *P. semifasciolatus* and *P. snyderi* was 0.12. Johns and Avise (1998) found that the mean genetic distances between different fishes within the same genus ranged from 0.02 to 0.36. Although these values range widely among fishes, the average value for the cyprinid fishes is 0.109 (Johns and Avise 1998). This suggests that *P. semifasciolatus* and *P. snyderi* can reasonably be regarded as two different species. Our argument was further supported by the phylogenetic analysis. Specimens of *P. semifasciolatus* and *P. snyderi* form two monophyletic groups with high statistical support (Fig. 3).

Although *P. semifasciolatus* was once alternately placed in the genera of *Barbus* (Günther 1868), *Capoeta* (Oshima 1919), and *Puntius* (Herre and Myers 1931), phylogenetic analysis revealed that *Barbus* and *Capoeta* are not included in the monophyletic clade of *P. semifasciolatus* and *P. snyderi*. These results suggest that *P. semifasciolatus* and *P. snyderi* share the most-recent common ancestor and cannot appropriately to be placed in either *Barbus* or *Capoeta*. The phylogenetic tree (Fig. 3) also reveals that the *Puntius* is a paraphyletic genus because *P. conchius* and *P. titteya* did not cluster with *P. semifasciolatus* and *P. snyderi*. Revision of these genera in the future is expected.



MrBayes: Posterior probability/ ML:Percentange bootstrap support

Fig. 3. Phylogenetic tree constructed by the Bayesian method with 2 X 10^6 times MCMC and maximum-likelihood method with 250 bootstrapping values.

Calibration of divergence time and biogeography of *Puntius* species in Taiwan

The evolutionary rate of the cytochrome b gene calibrated by first method was 0.368% per MY, which is slower than the 2% per MY evolutionary rate of birds (Randi 1996, Krajewski and King 1996) but close to the 0.294% per MY calculated by (Poh 2001). Krieger and Fuerst (2002) found that the evolutionary rate of cytochrome b gene in cyprinids was the slowest compared to that of Acipenseriformes, salmonids, percomorphs, and elopomorphs. It is worth noting that the sturgeon (Acipenseriformes) is considered a "living fossil" and possess a slow rate of molecular evolution (Krieger and Fuerst 2002). Curiously, the evolutionary rate of cytochrome b in cyprinid is slower than that of the sturgeon (Krieger and Fuerst 2002).

The estimation of divergence time between the *P. semifasciolatus* and *P. snyderi* is about 26.39 MYA which falls in the Oligocene. That is consistent with the fossil records that the present cyprinid fish genera such as the *Tinca*, *Rutilus*, and *Leuciscus* can be traced back to the Oligocene (Jefferies 1995, Yue et al. 2000). The results suggest that the speciation event between *P. semifasciolatus* and *P. snyderi* occurred in China not in Taiwan because the current island of Taiwan was formed by the Penglai Orogeny in the Pliocene about 5.2 MYA (Shen 1996). The fish later migrated from China to Taiwan across land bridges formed during subsequent ice ages.

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