Molecular Phylogeny and Genetic Differentiation of the *Tanakia himantegus* Complex (Teleostei: Cyprinidae) in Taiwan and China

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Chia-Hao Chang, Wen-Wen Lin, Yi-Ta Shao, Ryoichi Arai, Toshihiro Ishinabe, Takayoshi Ueda, Masaru Matsuda, Hitoshi Kubota, Feng-Yu Wang, Nian-Hong Jang-Liaw, and Hsiao-Wei Kao (2009) Molecular phylogeny and genetic differentiation of the *Tanakia himantegus* complex (Teleostei: Cyprinidae) in Taiwan and China. *Zoological Studies* **48**(6): 823-834. *Tanakia himantegus himantegus* is a subspecies endemic to Taiwan (referred as the Taiwanese *himantegus*), while *T. himantegus chii* is distributed in both Taiwan (referred as the Taiwanese *chii*) and China (referred as the Chinese *chii*). We analyzed the complete cytochrome (Cyt) *b* DNA sequences of 61 specimens of the *T. himantegus* complex (including the Taiwanese *chii*, Chinese *chii*, and Taiwanese *himantegus*) to infer their phylogeny, genetic differentiation, and historical demography. Both Bayesian and maximum-likelihood trees showed that the Taiwanese *chii*, Chinese *chii*, and Taiwanese *himantegus* are 3 monophyletic groups. Among them, the Taiwanese *chii* clustered with the Chinese *chii*. The average pairwise genetic distance (HKY + G) between the Taiwanese *chii* and Chinese *chii* was 6.8%, which is smaller than 10.8% (distance between the Taiwanese *chii* and Taiwanese *himantegus*) and 11.8% (distance between the Chinese *chii* and Taiwanese *himantegus*). The results suggest that the Taiwanese *chii* is phylogenetically closer to the Chinese *chii* than to the Taiwanese *himantegus*. Sequence analyses showed that the Taiwanese *chii* has smaller genetic diversity ($h = 0.771$, $\pi = 0.0014$) than the Chinese *chii* ($h = 0.927$, $\pi = 0.0087$) and Taiwanese *himantegus* ($h = 0.879$, $\pi = 0.0066$). The AMOVA revealed that about 92.8% of the genetic variance among sequences can be explained by differences among the 3 monophyletic groups (Taiwanese *chii*, Chinese *chii*, and Taiwanese *himantegus*). A unimodal mismatch distribution with a positively skewed distribution for the Taiwanese *chii* suggests that it has recently experienced sudden population expansions. Bimodal or ragged mismatch distributions for the Chinese *chii* and Taiwanese *himantegus* suggest that they are either admixtures of 2 expanding populations or stable populations. The origin of the Taiwanese *chii* is discussed based on the geographical history of Taiwan, records of fish collection, and phylogenetic analyses. http://zoolstud.sinica.edu.tw/Journals/48.6/823.pdf

Key words: Acheilognathinae, Cytochrome *b* gene, Mismatch distribution, Phylogeny.

Historical events play important roles in shaping the distribution and genetic structure of

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freshwater fish. Taiwan is an island located on the edge of the western Pacific Ocean. To the west, it is bounded by the Taiwan Strait, 160 km from China on the Asian mainland. The water depth in most of the Strait today does not exceed 100 m. Repeated sea level changes during the Pleistocene glacial-interglacial cycles brought about connections and disconnections of rivers of Taiwan and China (Boggs et al. 1979), which resulted in similarities and divergences of the freshwater fish fauna between Taiwan and China. Today, 63 species of primary freshwater fish have been recorded in Taiwan. Among them, about 34 species are endemic to Taiwan, and 29 species are common to both Taiwan and China (Chen and Fang 1999). Thus, freshwater fish in Taiwan and China provide favorable models for the study of genetic differentiation by dispersal and vicariance events.

Bitterlings are small cyprinid fish distributed in Asia and Europe and classified in the subfamily Acheilognathinae. They are characterized by spawning their eggs into freshwater mussel gills (Smith et al. 2004). Although 7 genera have been nominated in the Acheilognathinae (Arai 1988), 3 genera (*Tanakia*, *Acheilognathus*, and *Rhodeus*) are currently recognized based on morphology, phylogenetic relationships by mitochondrial 12S ribosomal DNA sequences and diploid chromosome number (Arai 1982, Arai and Akai 1988, Okazaki et al. 2001). Among these 3 genera, *Tanakia* is characterized by having 48 diploid chromosomes and 2 long barbels. Three species are distributed in Japan (*T. tanago*, *T. lanceolata*, and *T. limbata*), 4 species in Korea (*T. signifier*, *T. koreensis*, *T. somjinensis*, and *T. lanceolata*), 1 species and 1 subspecies in China (*T. lanceolata* and *T. himantegus chii*), and 1 subspecies in Taiwan (*T. h. himantegus*) (Ueda et al. 2006).

In the *T. himantegus* complex, 2 taxa were reported from Taiwan and China: *T. h. himantegus* was originally described as *Achilognathus himantegus* by Günther (1868) based on 4 specimens from Taiwan, and *T. h. chii* was described as *A. chii* by Miao (1934) based on 1 specimen collected from Chin-kiang, Jiangsu Province, China. When Miao nominated *A. chii*, this species was not compared to *A. himantegus*, and diagnostic characters of *A. chii* and *A. himantegus* were not described. Therefore, *A. chii* was once a synonym of *Paracheilognathus himantegus* (= *T. himantegus*) (Woo 1964, Chen

et al. 1998). These 2 nominal taxa were classified into the genus *Tanakia* by Arai and Akai (1988). Arai and Kato (2003) classified *T. himantegus* into 2 subspecies, *T. h. himantegus* and *T. h. chii*.

A nuptial male of *T. h. himantegus* has a red band on the dorsal fin, red patches on the operculum and pectoral fin, and a red iris (Figs. 1A, B), while that of *T. h. chii* has a yellowish-white band on the dorsal fin and a non-red iris (Figs. 1C-F) (Arai 2008). Because these 2 taxa can only be identified by the male nuptial color, it is difficult to determine whether specimens deposited in museums, for example, specimens from Shandong, Fujian, and Zhejiang Provinces, China (Chu 1984, Mao and Xu 1991, Chen and Zhou 1997), are *T. h. chii* or *T. h. himantegus*. A taxonomic review of the *T. himantegus* complex has not been published to date, and relationships between *T. h. himantegus* and *T. h. chii* are not resolved. Chen and Chang (2005) described *T. himantegus* as being widely distributed in Taiwan, but *T. chii* is restricted to some ponds or lowland rivers in northern Taiwan. However, as far as we know, distributions of *T. h. chii* in Taiwan are restricted to 2 localities, i.e., 1 small pond and 1 small lowland river in northern Taiwan, and the Taiwanese *chii* was not discovered in Taiwan until 2006. It is questionable whether the Taiwanese *chii* is native to Taiwan.

This study attempted to infer phylogenetic relationships of the *T. himantegus* complex in Taiwan and China. Specifically, the hypotheses tested include hypothesis 1: the Taiwanese *chii* is phylogenetically closer to the Taiwanese *himantegus* than to the Chinese *chii*; hypothesis 2: the Taiwanese *chii* is phylogenetically closer to the Chinese *chii* than to the Taiwanese *himantegus*; and hypothesis 3: the Chinese *chii* is phylogenetically closer to the Taiwanese *himantegus* than to the Taiwanese *chii*. Phylogenetic trees were constructed. In addition, haplotype diversity, nucleotide diversity, analysis of molecular variance (AMOVA), Fu's F_S test, and mismatch distribution were calculated to infer their genetic differentiation and historical demography.

MATERIALS AND METHODS

Sampling specimens

Tanakia h. himantegus was collected from 10 locations in Taiwan (referred to as the Taiwanese *himantegus*) (Table 1, Fig. 2): Ilan (locality 1; 1

specimen), Chinlung Lake (Hsichih) (locality 3; 12 specimens), Bade (locality 4; 3 specimens), and Sanyi (locality 6; 1 specimen) of northern Taiwan; Wujih (locality 7; 3 specimens) of central Taiwan; Danei (locality 8; 3 specimens) and Meinong (locality 9; 1 specimen) of southern Taiwan; and Shoufeng (locality 10; 3 specimens), Yuli (locality 11; 3 specimens), and Taitung (locality 12; 3 specimens) of eastern Taiwan. Divisions of

southern, central, northern, and eastern Taiwan followed Chen and Fang (1999). Specimens of *T. h. chii* were collected in Shanghai (locality 13, 11 specimens) in China (referred as the Chinese *chii*) and Tsuichih (locality 2, 11 specimens) and Taoyuan (locality 5, 4 specimens) in northern Taiwan (referred as the Taiwanese *chii*). Among the 12 sampling localities in Taiwan, Tsuichih and Chinlung Lake are in the watershed of the Keelung
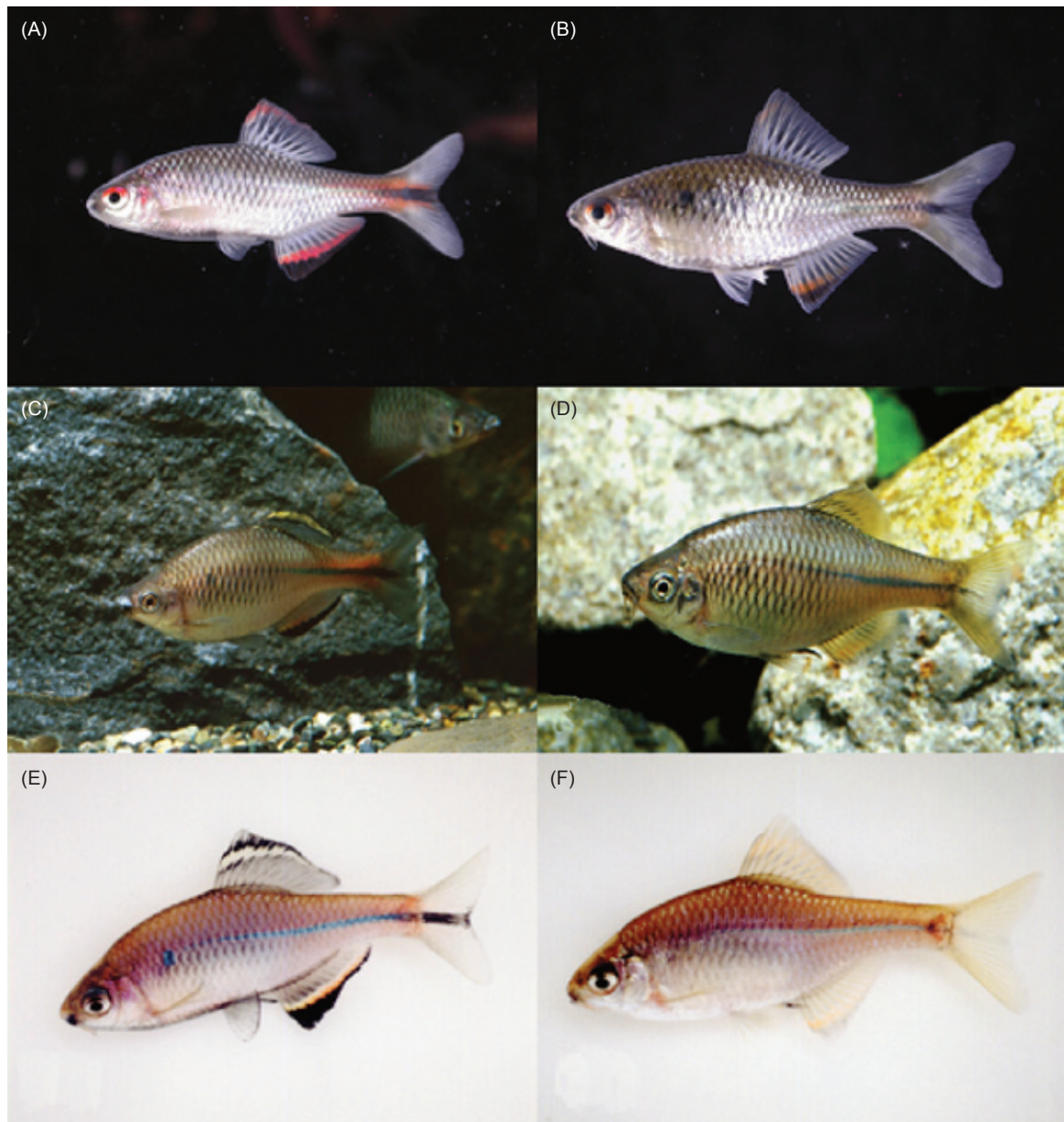

Fig. 1. Photos of *Tanakia himantegus chii* (referred to as the Taiwanese *chii* or Chinese *chii* based on sampling localities) and *T. h. himantegus* (referred to as the Taiwanese *himantegus*). (A) Male *T. h. himantegus* from Bade, Taiwan; (B) female *T. h. himantegus* from Bade, Taiwan; (C) male *T. h. chii*, from Tsuichih, Taiwan; (D) female *T. h. chii* from Tsuichih, Taiwan; (E) male *T. h. chii*, from Shanghai, China; (F) female *T. h. chii* from Shanghai, China.

River, Bade and Taoyuan are in the watershed of the Dahan River, and Taitung and Yuli are in the watershed of the Siouguluan River. Shanghai is in the watershed of the Yangtze River. Specimens of *T. lanceolata* (locality 14, 2 specimens), *T. limbata* (locality 14; 2 specimens), and *T. tanago* (locality 14, 1 specimen) were sampled from Japan, and *T. koreensis* (locality 15, 1 specimen) was collected from Korea. In this study, 5 of 7 *Tanakia* species were included.

DNA extraction and sequencing of the cytochrome (Cyt) *b* gene

Total DNA was extracted by a Gentra-DNA extraction kit (Gentra, Minneapolis, MN, USA). The complete mitochondrial Cyt *b* gene was amplified by a polymerase chain reaction (PCR). The primers, Cyto1 5'-TAGTTCAACTACAAGAAC AATT-3' and Cyto2 5'-TAGGCTAAGCTACTAGG GCA-3', were designed from a conserved region of complete mitochondrial (mt)DNA sequences of *Rhodeus* uyekii (NC_007885) (Kim et al. 2006), *R. ocellatus* (NC_011211) (He et al. 2008), *R. ocellatus kurumeus* (NC_008642) (Saitoh et al. 2006), and *A. typus* (NC_008668) (Saitoh et al. 2006). Each 100 μl of the PCR contained about 10 ng template DNA, 10 μl 10× reaction buffer,

8 μl dNTP mix (2.5 mM dNTP each), 25 μmol of each specific primer, 2.5 units of *Taq* polymerase (TaKaRa, Kyoto, Japan), and distilled water. Thermal cycling began with 1 cycle of 94°C for 4 min, and subsequently 35 cycles of denaturation at 94°C for 1 min, annealing at 55-65°C for 1 min, and extension at 72°C for 1 min, followed by a final extension step at 72°C for 10 min. PCR products were purified with a PCR DNA Fragments Extraction Kit (Geneaid, Taichung, Taiwan). Approximately 50 ng of a purified DNA fragment was used in the cycle sequencing with the same primers as described above using the ABI PRISM BigDye sequencing kit (PE Applied Biosystems, Foster City, CA, USA) protocol. Reaction products were electrophoresed on an ABI Model 3100 vers. 3.7 automated sequencer (Applied Biosystems). Sequences from 2 directions of Cyt *b* were assembled using the BioEdit program vers. 5.0.9 (Hall 1999). The Cyt *b* DNA sequences of the specimens were deposited in GenBank under the accession numbers DQ178349-DQ178389, EU707378-EU707392, and EU750825-EU7508333 (Table 1). The sequence of *Zacco platypus* (AY245071) (Perdices et al. 2004) was downloaded from GenBank.

Table 1. Locality number, species, sampling locality and accession numbers of *Tanakia* specimens in this study

Locality no.	Species	No. of samples	Locality	Accession no.
1	*T. h. himantegus*	1	Ilan, Taiwan	DQ178359
2	*T. h. chii*	11	Tsuichih, Hsichih, Taiwan	DQ178375-DQ178385
3	*T. h. himantegus*	12	Chinlung Lake, Hsichih, Taiwan	DQ178355-DQ178357 EU750825-EU750833
4	*T. h. himantegus*	3	Bade, Taiwan	DQ178349-DQ178351
5	*T. h. chii*	4	Taoyuan, Taiwan	EU707384-EU707387
6	*T. h. himantegus*	1	Sanyi, Taiwan	DQ178358
7	*T. h. himantegus*	3	Wujih, Taiwan	DQ178367-DQ178369
8	*T. h. himantegus*	3	Danei, Taiwan	DQ178364-DQ178366
9	*T. h. himantegus*	1	Meinong, Taiwan	DQ178360
10	*T. h. himantegus*	3	Shoufeng, Taiwan	DQ178361-DQ178363
11	*T. h. himantegus*	3	Yuli, Taiwan	DQ178352-DQ178354
12	*T. h. himantegus*	5	Taitung, Taiwan	EU707388-EU707392
13	*T. h. chii*	11	Shanghai, China	DQ178370-DQ178374 EU707378-EU707383
14	*T. lanceolata*	2	Japan	DQ178386-DQ178387
14	*T. limbata*	2	Japan	DQ178388-DQ178389
14	*T. tanago*	1	Japan	AB485774
15	*T. koreensis*	1	Korea	FJ789986

Sequence analyses

Base frequencies of sequences (including

Cyt *b* sequences of the Taiwanese *himantegus*, Chinese *chii*, Taiwanese *chii*, Japanese *T. lanceolata*, *T. limbata*, and *T. tanago*, and Korean *T.*
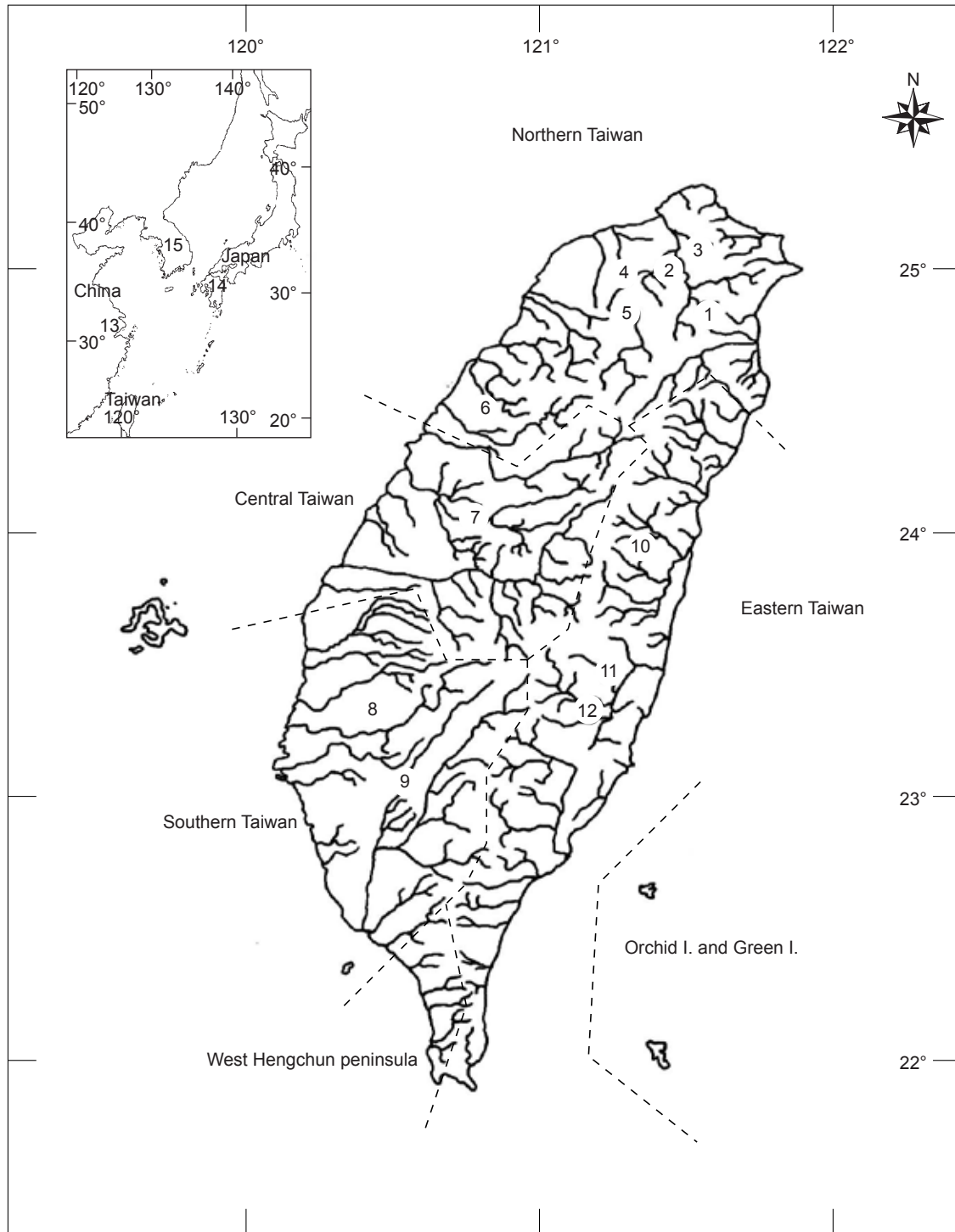

Fig. 2. Map of sampling localities. Numbers on the map correspond to those in table 1. **1**, Ilan; **2**, Tsuichih, Hsichih; **3**, Chinlung Lake, Hsichih; **4**, Bade; **5**, Taoyuan; **6**, Sanyi; **7**, Wujih; **8**, Danei; **9**, Meinong; **10**, Shoufeng; **11**, Yuli; **12**, Taitung; **13**, Shanghai; **14**, Japan; **15**, Korea.

koreensis were calculated using Dambe software (Xia and Xie 2001). Numbers of invariable, variable, singleton variable, and parsimoniously informative sites of Cyt *b* sequences were calculated using DnaSP vers. 4.50.2 software (Rozas and Rozas 2003). Tajima's *D*-test (Tajima 1989), which tests the conformity of DNA sequence evolution to neutrality, was also performed using DnaSP software.

Reconstruction of phylogenetic trees

Phylogenetic trees were reconstructed using the Bayesian inference (BI) method implemented in MrBayes (Huelsenbeck and Ronquist 2001) and the maximum-likelihood (ML) method implemented in PAUP 4.10b (Swofford 2001). Sequence alignment was conducted by using ClustalW (Thompson et al. 1994). The best-fit model of HKY (Hasegawa et al. 1985) with a gamma distribution (G) (Yang 1994) of 0.2712, and base frequencies of A = 0.2674, C = 0.2875, G = 0.1570, and T = 0.2882 were selected by Modeltest 3.7 (Posada and Crandall 1998) with the Akaike information criterion (AIC). The model was incorporated in the BI and ML analyses. MrBayes 3.1.2 (Huelsenbeck and Ronquist, 2001) was used for the BI analysis. In the BI analysis, random starting trees were used. In total, 2×10^6 generations of Markov chains were run. Trees were saved every 100 generations which resulted in 20,000 trees in the initial samples. The stationary phase of log-likelihood was reached within 5×10^5 generations. Thus burn-in (the number of initial trees discarded) was set to 5000. Majority rule consensus trees were generated from the remaining samples (15,000 trees), and the percentage of samples recovering any particular clade represented the clade's posterior probability (Huelsembeck and Ronquist 2001). In the ML analysis, a heuristic search was conducted. The starting tree for branch-swapping was from stepwise addition. Nodal support of the ML tree was estimated by 1000 bootstraps. In these 2 analyses, *Zacco platypus* was chosen as the outgroup in accordance with Okazaki et al. (2001).

In order to investigate the genetic structures within and among the Taiwanese *himantegus*, Taiwanese *chii*, and Chinese *chii*, haplotype networks were reconstructed, based on the statistical parsimony criterion (Templeton et al. 1992) implemented in TCS 1.21 (Clement et al. 2000).

Population variation, differentiation, and expansion

The haplotype number, haplotype diversity (*h*), nucleotide diversity (π), Tajima's *D*-test, and F_{ST} of each group (lineages) were calculated using DnaSP 4.50.2. Numbers of base substitutions per site from the average of overall sequence pairs among the 3 groups were calculated with the HKY (Hasegawa et al. 1985) + G model (Yang 1994) using PAUP 4.10b (Swofford 2001). In order to analyze the molecular variance at different levels of hierarchical subdivisions (among groups, among populations, and within population) and population expansions, analysis of molecular variance (AMOVA), the distributions of pairwise differences between sequences (mismatch distribution), and Fu's F_S test (Fu 1997) were conducted using the program Arlequin 3.11 (Excoffier et al. 2005) with 1000 permutations.

RESULTS

Characterization of Cyt *b* in *Tanakia*

The length of Cyt *b* in all 5 species of *Tanakia* was 1140 bp. Among them, 802, 338, 39, and 299 bp were invariable, variable, singleton variable, and parsimoniously informative sites, respectively. The majority of variable and phylogenetically informative sites of Cyt *b* were 3rd codon position substitutions (76.9% and 83.9%, respectively), and the lower proportions were 1st (17.4% and 14.0%) and 2nd (5.6% and 2.0%) codon position substitutions. Empirical base frequencies were A = 26.8%, C = 25.9%, G = 15.9%, and T = 31.3%, with an anti-G bias, a characteristic of the mitochondrial genome (Cantatore et al. 1994). Chi-square test showed that the heterogeneity in nucleotide frequencies among operational taxonomic units (OTUs) was not significant (χ^2 = 80.27, *d.f.* = 192, *p* = 1.0). A neutrality test for the entire data set showed that Tajima's *D* value (-0.54221) did not significantly deviate from 0 (*p* > 0.1).

Phylogenetic analysis

The Bayesian tree of Cyt *b* DNA sequences is shown in figure 3. Levels of statistical support of posterior probabilities (in the BI) and bootstrap values (in the ML analysis) are marked on the branches for values > 70%. The tree showed

that the Taiwanese *himantegus*, Taiwanese *chii*, and Chinese *chii* formed 3 different monophyletic groups (clades) that were supported by 100% posterior probability in the BI and 94%-96% bootstrap values in the ML analysis. Among them, the Taiwanese *chii* was clustered with the Chinese *chii*; however, the statistical support for the nodes was 71% in the posterior probability and < 70% for the bootstrap value. *Tanakia tanago* was clustered with the *T. himantegus* complex. For the other 3 *Tanakia* species, *T. koreensis* was clustered with *T. limbata*. These 2 species were further clustered with *T. lanceolata*, and they were at the basal position of the tree compared to the positions of *T. tanago* and the *T. himantegus* complex (Fig. 3).

Genetic differentiation among the 3 groups

In 61 individuals of *T. h. himantegus* and *T.*

h. chii, 34 haplotypes were observed. Among them, 19, 8, and 7 haplotypes were detected in the Taiwanese *himantegus* (35 individuals), Chinese *chii* (11 individuals), and Taiwanese *chii* (15 individuals), respectively. Haplotype diversity values (h) ± the standard deviation (SD) were 0.879 ± 0.050 for the Taiwanese *himantegus* (35 individuals), 0.927 ± 0.066 for the Chinese *chii* (11 individuals), and 0.771 ± 0.010 for the Taiwanese *chii* (15 individuals). Nucleotide diversities (π) ± the SD were 0.00665 ± 0.00075, 0.00874 ± 0.00187, and 0.00140 ± 0.00042 for the Taiwanese *himantegus*, Chinese *chii*, and Taiwanese *chii*, respectively.

Pairwise genetic distances of Cyt *b* sequences calculated with the HKY (Hasegawa et al. 1985) + G (Yang 1994) model are shown in table 2. Genetic distances among 7 species of *Tanakia* fish ranged 6.8%-41.9%. Genetic
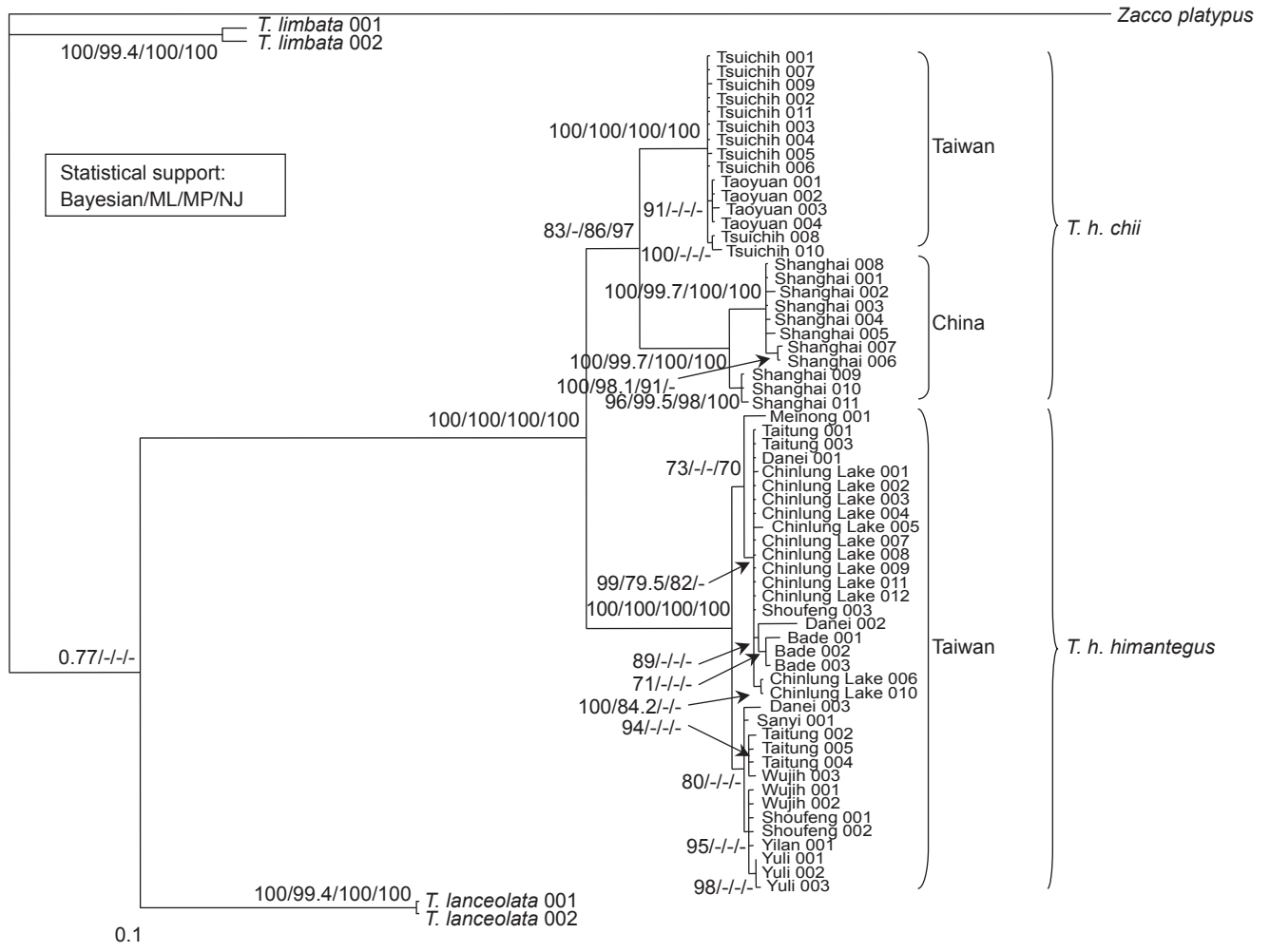

Fig. 3. Phylogenetic tree of the *Tanakia himantegus* complex in Taiwan and China inferred from mitochondrial cytochrome *b* DNA sequences. The tree was constructed by the Bayesian method. Statistical support is marked on the branches when > 70%.

distances between the Taiwanese *himantegus* and Chinese *chii*, between the Taiwanese *himantegus* and Taiwanese *chii*, and between the Taiwanese *chii* and Chinese *chii* were 11.8%, 10.8%, and 6.8%, respectively.

F_{ST} values between the Taiwanese *himantegus* and Taiwanese *chii*, between the Chinese *chii* and Taiwanese *chii*, and between the Taiwanese *himantegus* and Chinese *chii* were 0.946, 0.905, and 0.905, respectively. AMOVA showed that the variance among the 3 groups (Taiwanese *himantegus*, Taiwanese *chii*, and Chinese *chii*), among populations, and within populations were 92.81%, 4.75%, and 2.44%, respectively. Φ_{CT} (fixation in a specific group of subpopulations relative to the total population), Φ_{SC} (fixation in a subpopulation relative to a specific group of subpopulations), and Φ_{ST} (fixation in a subpopulation relative to the total) were 0.9281 ($p < 0.01$), 0.3396 ($p < 0.001$), and 0.9525 ($p < 0.001$), respectively (Table 3).

Haplotype network

As shown in figure 4, no haplotype was shared by the 3 groups. Eighty-one substitutions separated the Taiwanese *himantegus* and Taiwanese *chii*, 79 substitutions separated the Taiwanese *himantegus* and Chinese *chii*, and 48 substitutions separated the Taiwanese *chii* and Chinese *chii*. Among them, the Taiwanese *chii* was characterized by a short connection among haplotypes, i.e., 1 substitution, while there were more than 10 substitutions between haplotypes of the Taiwanese *himantegus* and Chinese *chii*.

Historical demography

Mismatch distribution for the Taiwanese *himantegus* (sum of squared deviations (SSD) = 0.049, $p = 0.156$; R, Harpending's raggedness index = 0.046, $p = 0.392$) and Chinese *chii* (SSD = 0.081, $p = 0.249$; $R = 0.129$, $p = 0.540$) were bimodal or ragged (Figs. 5A, C), while the mismatch distribution of the Taiwanese *chii* (SSD = 0.028, $p = 0.127$; $R = 0.204$, $p = 0.133$) was unimodal with a positively skewed distribution (Fig. 5B). Values of Fu's F_S test were -25.653 for the Taiwanese *himantegus*, -3.40×10^{-39} for the Taiwanese *chii*, and -10.352 for the Chinese *chii*. These values were negative and significantly ($p < 0.05$) differed from 0.

Table 2. Percentage of pairwise genetic distances between *Tanakia* fish calculated with the HKY (Hasegawa et al. 1985) + G (Yang 1994) model

Species or subspecies	1	2	3	4	5	6	7
1. *T. lanceolata*	-	26.4	30.3	25.5	39.9	35.9	41.9
2. *T. limbata*	-	-	29.8	16.1	41.4	38.4	41.6
3. *T. tanago*	-	-	-	29.7	34.5	27.4	28.2
4. *T. koreensis*	-	-	-	-	39.4	33.2	39.4
5. Taiwanese *himantegus*	-	-	-	-	-	10.8	11.8
6. Taiwanese *chii*	-	-	-	-	-	-	6.8
7. Chinese *chii*	-	-	-	-	-	-	-

Table 3. Analysis of molecular variance (AMOVA) among populations of 3 clades, the Taiwanese *himantegus*, Taiwanese *chii*, and Chinese *chii*, based on analyses of the cytochrome *b* sequences

Source of variation	d.f.	Percent (%) of total variance	Fixation indices (Φ)	p
Among clades	2	92.81	0.9281	0.0029
Among clades within groups	5	2.44	0.3396	< 0.0001
Within populations	53	4.75	0.9525	< 0.0001

DISCUSSION

The BI and ML trees of Cyt *b* DNA sequences showed that the Taiwanese *chii* and Chinese *chii* were clustered together (Fig. 3). The pairwise genetic distance between the Taiwanese *chii* and Chinese *chii* (6.8%) was smaller than distances between the Taiwanese *chii* and Taiwanese *himantegus* (10.8%), and between the Chinese *chii* and Taiwanese *himantegus* (11.8%). Based on the congruent tree topology inferred from the BI and ML analyses, hypothesis 2 that the Taiwanese *chii* is phylogenetically closer to the Chinese *chii* than to the Taiwanese *himantegus* is better supported.

In the Taiwanese *himantegus*, specimens from central northern, western, central, and eastern Taiwan were clustered together (Fig. 3). This result is not consistent with previous studies (Wang 1997, Yeh 1997, Hsu 1999) that animals in eastern Taiwan are genetically divergent from animals in western Taiwan. Because rivers in western and eastern Taiwan are separated by the Central Mountain Range, the absence of genetic differentiation of the Taiwanese *himantegus* may have resulted from reciprocal introductions between eastern and western populations of the Taiwanese *himantegus* by human activities.

Pairwise genetic distances among the 7 *Tanakia* species ranged 6.8%-41.9% (Table 2). Among them, the distance between the Taiwanese *chii* and Chinese *chii* was the smallest (6.8%), which supports the Taiwanese *chii* and Chinese
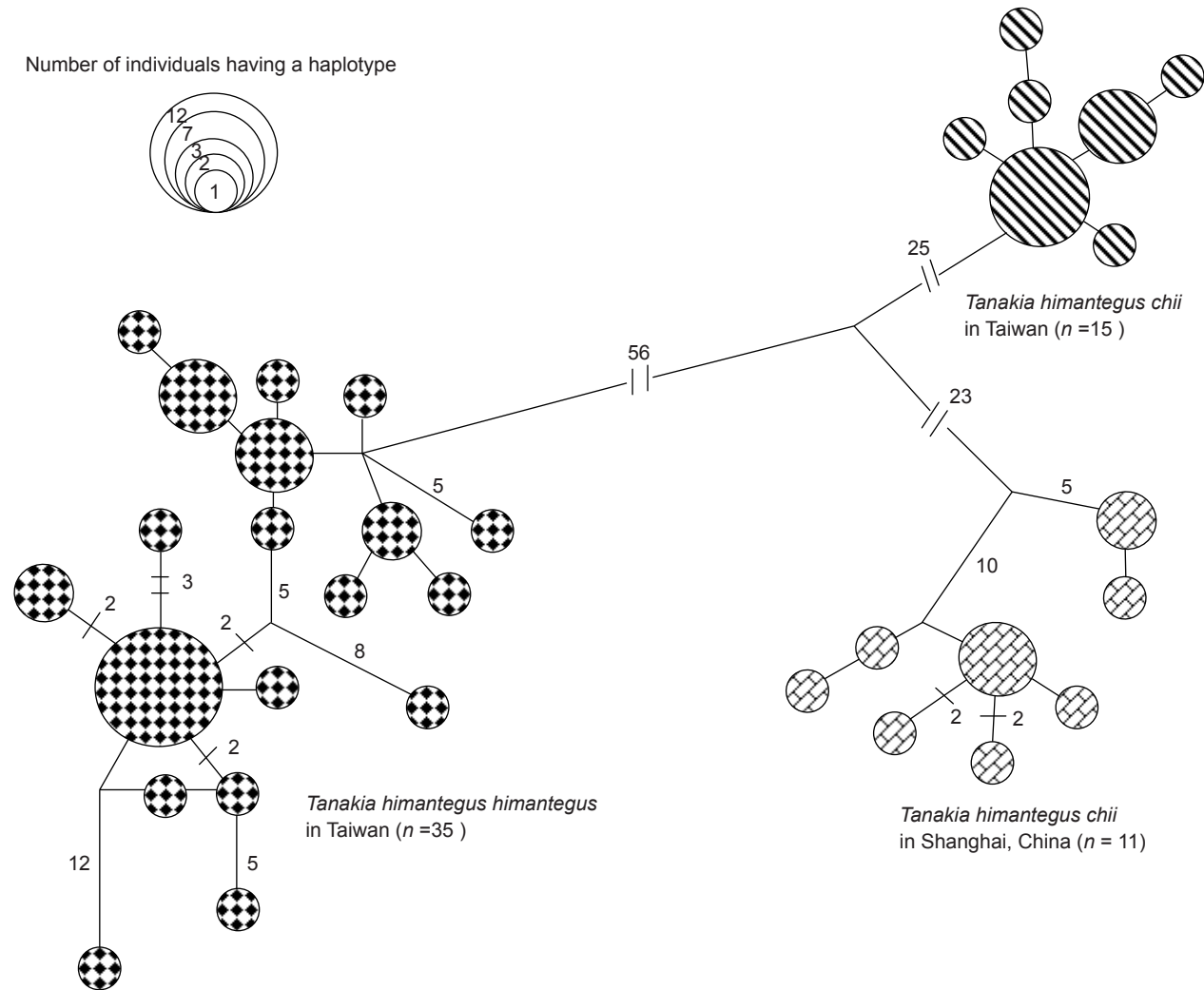

Fig. 4. Parsimonious network of the *Tanakia himantegus* complex reconstructed by the program TCS 1.21 (Clement et al. 2000). A line connecting 2 haplotypes represents 1 nucleotide substitution. Greater than 2 substitutions are shown by numbers.
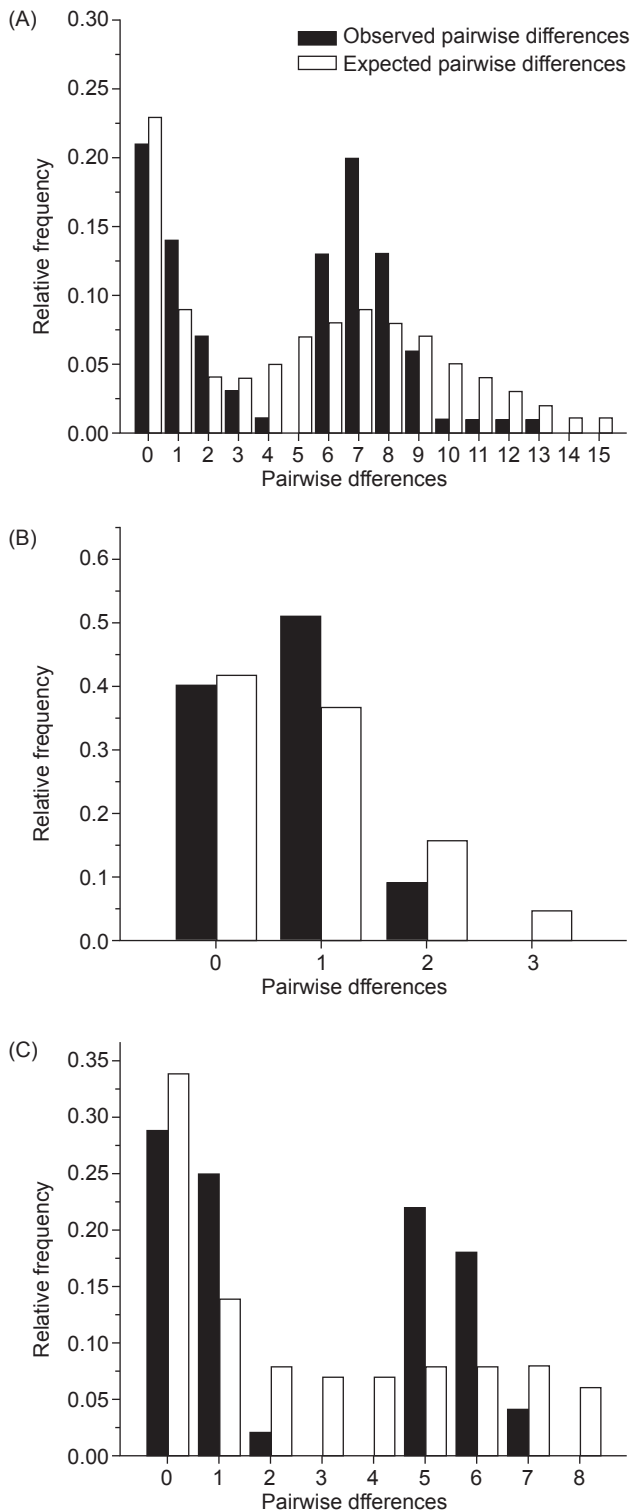
Fig. 5. Observed (black bars) and expected (white bars) mismatch distributions under the sudden expansion model for cytochrome *b* sequence haplotypes in (A) the Taiwanese *himantegus* (sum of squared deviations (SSD) = 0.049, *p* = 0.156; *R* = 0.046, *p* = 0.392), (B) Taiwanese *chii* (SSD = 0.028, *p* = 0.127; *R* = 0.204, *p* = 0.133), and (C) Chinese *chii* (SSD = 0.081, *p* = 0.249; R = 0.129, *p* = 0.540).

chii having a closer phylogenetic relationship than other *Tanakia* fish. With the exception of the nuptial coloration of the male, *T. h. chii* (Taiwanese *chii* and Chinese *chii*) and *T. h. himantegus* (Taiwanese *himantegus*) are indistinguishable by morphological characters. The pairwise genetic distance between them was the smallest among *Tanakia* fish. Therefore, currently there is not enough evidence to classify *T. h. himantegus* (Taiwanese *himantegus*) and *T. h. chii* (Taiwanese and Chinese *chii*) as 2 different species. We tentatively classify them as different subspecies. For the coexistence of 2 subspecies of *T. himantegus* in Taiwan, we propose that Taiwanese *T. h. chii* was recently artificially introduced from China, because the Taiwanese *T. h. chii* was not discovered in Taiwan until 2006, and currently it can only be found in 2 localities in Taiwan. The smallest genetic differentiation among the *T. himantegus* complex and its star-like haplotype network support this hypothesis.

The divergence times between lineages were calculated by the formula T = K/2r, where K is the number of substitutions per site between 2 homologous sequences, and r is the number of substitutions per site per year (Graur and Li 1991). If the Cyt *b* gene in bitterlings evolves at a similar rate to other cyprinids, i.e., 0.76%-1.25% per nucleotide per million years (Zardoya and Doadrio 1999, Machordom and Doadrio 2001, Durand et al. 2002, Ketmaier et al. 2004), estimated divergence times are 4.72-7.76 million years ago (Mya) between the Taiwanese *himantegus* and Chinese *chii*, 4.32-7.11 Mya between the Taiwanese *himantegus* and Taiwanese *chii*, and 2.72-4.47 Mya between the Taiwanese *chii* and Chinese *chii*. This is consistent with fossil records of many extant cyprinid fish such as *Rhodeus*, *Gobio*, and *Aspius* that can be traced back to the Miocene (Frickhinger 1995, Chen et al. 1998).

Because the estimated divergence time between *T. h. chii* and *T. h. himantegus* (4.32-7.76 Mya) is earlier than the estimated time that the island of Taiwan emerged from the sea surface (3-5 Mya, Chemenda et al. 2001) and the earliest connection of Taiwan with the Asian mainland was estimated to be 1-2 Mya (Lin 1963, Teng 1990), we propose that *T. h. himantegus* and *T. h. chii* were differentiated in China. After *T. h. himantegus* dispersed to Taiwan, it became extinct in China. A similar inference was also proposed for *Puntius snyderi* and *P. semifasciolatus* in which *P. snyderi* is a species endemic to Taiwan and *P. semifasciolatus* is distributed in both China and

Taiwan (Chang et al. 2006). These 2 species were proposed to have differentiated in China. After *P. snyderi* and *P. semifasciolatus* dispersed to Taiwan, *P. snyderi* became extinct in China (Chang et al. 2006).

Sequence analysis indicated that the genetic diversity of the Taiwanese *chii* (*h* = 0.771, π = 0.0014) was smaller than those of the Taiwanese *himantegus* (*h* = 0.879, π = 0.0066) and Chinese *chii* (*h* = 0.927, π = 0.0087). A large *h* and small π were also observed in an introduced cyprinid, *Z. platypus*, in Taiwan (Ma et al. 2006). A unimodal mismatch distribution with a positively skewed distribution and negative value of Fu's F_S test suggest that the Taiwanese *chii* has experienced a recent population expansion (Rogers and Harpending 1992) (Fig. 5B). The bimodal or ragged mismatch distributions of the Taiwanese *himantegus* and Chinese *chii* suggest that they are either admixtures of 2 expanding populations or a stable population (Figs. 5A, C) (Rogers and Harpending 1992). The phylogenetic analyses showed that both the Taiwanese *himantegus* and Chinese *chii* comprise 2 sublineages in the clades, which supports the hypothesis of the existence of 2 expanding populations for the Taiwanese *himantegus* and Chinese *chii*.

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