

## Phylogeography of the Taiwanese Endemic Hillstream Loaches, *Hemimyzon formosanus* and *H. taitungensis* (Cypriniformes: Balitoridae)

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**Tzi-Yuan Wang, Te-Yu Liao, and Chyng-Shyan Tzeng (2007)** Phylogeography of the Taiwanese endemic hillstream loaches, *Hemimyzon formosanus* and *H. taitungensis* (Cypriniformes: Balitoridae). *Zoological Studies* 46(5): 547-560. Variations in nucleotide sequences within the mitochondrial control region were used to determine the paleogeography of speciation and diversification of 2 balitorids endemic to Taiwan. Examination of 11 populations of *Hemimyzon formosanus* and 5 populations of *H. taitungensis* respectively revealed 23 and 11 haplotypes within the mitochondrial control regions. Utilizing the neighbor-joining method and maximum-parsimony trees, we showed the presence of 3 groups and 2 subgroups in *H. formosanus*, and 1 group in *H. taitungensis*. The nested clade analysis, a method with a higher resolution, revealed that the 1 group of *H. taitungensis* could be further divided into 2 subgroups on the minimum spanning network. The nested clade analysis predicted the evolutionary divergence of populations in *H. formosanus* due to past fragmentation; furthermore, dispersion among populations of *H. taitungensis* was caused by long-distance colonization. The moderate gene flow and low genetic divergence within the mitochondrial control region suggest that local range expansion and recent colonization occurred in *H. formosanus* in west-central Taiwan and in *H. taitungensis* in eastern Taiwan. Our study showed that one of the major influences on the speciation of both western *H. formosanus* and eastern *H. taitungensis* was the Central Mountain Range of Taiwan. Moreover, deep genetic divergence and morphological differences suggest new phylogenetic species exist within *H. formosanus*.  
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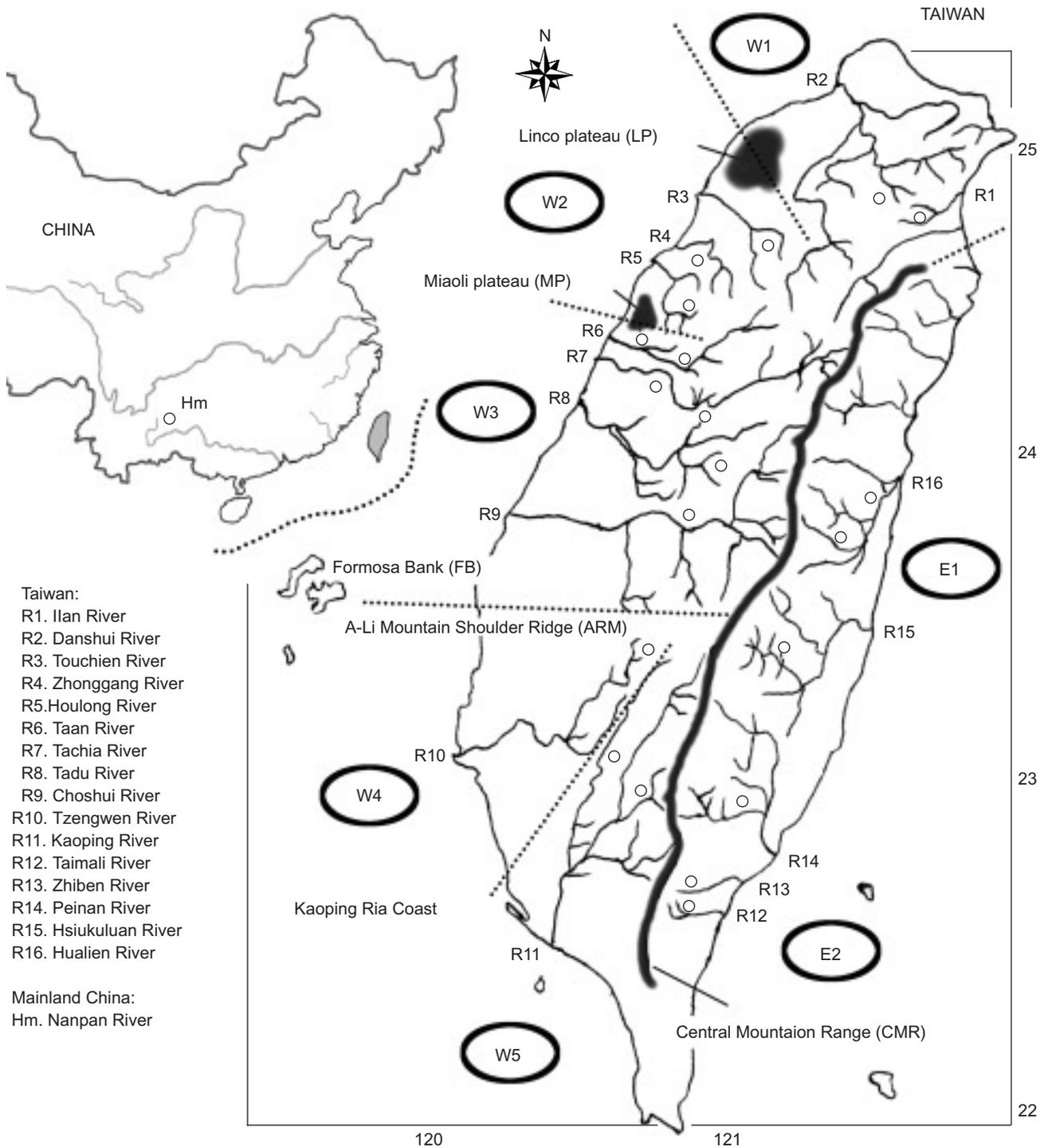
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Geographical barriers, such as mountains, may influence the speciation and isolate populations from one another. On the other hand, animals can easily disperse in a region without such barriers. Determining when dispersal and vicariance have alternately influenced colonization and subdivision of a species by historical biogeography is quite complicated, but these events frequently occur in island systems (Emerson 2002, Wiens and Donoghue 2004, Tzeng et al. 2006). Mitochondrial phylogeography can provide important insights into evolutionary patterns of popula-

tion fragmentation and gene flow and offers perspective on speciation, diversification, and colonization of species (Avice 1994, Avice and Wollenberg 1997).

The Central Mountain Range (CMR) is the most important natural barrier in Taiwan (Fig. 1), and it separates many vertebrates and invertebrates such as shrimps, crabs, fishes, frogs, and lizards into 2 distinct groups or sister species (Chen 1969, Tzeng 1986, Yang et al. 1994, Chang and Liu 1997, Chou and Lin 1997, Yeh 1997, Toda et al. 1998, Lin et al. 2002, Liu 2006, Liu et al.

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**Fig. 1.** Sampling locations of *Hemimyzon* species. The circles represent sampling localities. From figure 3, two rivers are located in region W1 (R1 and R2) and 3 are in region W2 (R3, R4, and R5). Region W3 consists of 4 rivers (R6, R7, R8, and R9). The Tzengwen River is located in region W4 (R10), and the Kaoping River is in region W5 (R11). Two (R12 and R13) and 3 rivers (R14, R15, and R16) are respectively located in regions E2 and E1 of eastern Taiwan. Specimens of *H. megalopseos* were collected from the Nanpan River of Yunnan Province, China. More-detailed information is given in table 1.

2007). Like those species, the geographical distributions of 2 different hillstream loaches are also divided by the CMR barrier; however, their mitochondrial phylogeography offers a different population history. The benthic, loach-like fish of the genus, *Hemimyzon* (Cypriniformes: Balitoridae), can only survive in highly oxygenated, non-polluted, upper reaches of rivers (Hora 1932, Chen 1980, Tzeng and Shen 1982). The distribution of *Hemimyzon formosanus* (Boulenger) is mainly in rivers of western Taiwan (Tzeng and Shen 1982). On the other hand, *H. taitungensis* Tzeng and Shen, the sister species of *H. formosanus*, is mainly located in eastern Taiwanese rivers. The ability to adapt to swift currents allows *H. taitungensis* to dominate other freshwater fishes in the upstream regions of eastern rivers (Tzeng and Shen 1982, Tzeng 1986).

Lin (1957) proposed a number of barriers, including the CMR, which were thought to have existed during previous ice ages. For example, the Miaoli Plateau formed about 300,000 yr ago, and the Formosa Bank, which is connected to Ali Mt., are both secondary barriers located in western Taiwan (Lin 1957, Tzeng 1986). The Formosa Bank, the land bridge between central Taiwan and the Nan Mountain range in southern China (Lin 1957), formed during recent ice ages due to lowering of sea levels. The formation of a land bridge led to the separation of the Northern and Southern Rivers (Boggs et al. 1979). These barriers and river systems, like the CMR, have caused various speciation, diversification, and colonization events among animal species.

Two modern speciation concepts redefine “species-level” taxa (Cracraft 1983, Nixon and Wheeler 1990, Avise 1994, Avise and Wollenberg 1997). The biological species concept (BSC) states that a species is a reproductively isolated population, while the phylogenetic species concept (PSC) defines a species as a group with monophyletically recognizable populations. Evolutionary history and reproductive ties are 2 related aspects of both concepts (Avise and Wollenberg 1997). Thus, by understanding the phylogeography of species, one can distinguish the relationships between speciation and population genetics.

According to the species composition and distribution of the freshwater fish fauna of Taiwan, Tzeng (1986) classified several major biogeographical zones: eastern, northwestern, and southwestern zones and a central intermediate zone

(Fig. 1). However, recent phylogeographic patterns have shown some different aspects in comparison to that of Tzeng (Wang et al. 1999, Wang et al. 2000, Wang et al. 2004). For example, *Varicorhinus barbatulus* (Pellegrin) was reported to be the earliest widespread fish in both western and eastern Taiwan (Tzeng 1986), but a recent phylogeographic analysis based on molecular data suggests that eastern populations of *V. barbatulus* were more recently colonized from southern populations (Wang et al. 2004). The freshwater fish fauna suggests that most animals may have colonized Taiwan from northern and southern regions then migrated to central Taiwan (Oshima 1923, Tzeng 1986). However, mitochondrial (mt)DNA analysis indicated that the colonization routes of *Acrossocheilus paradoxus* (Gunther) were dispersals from central Taiwan to northern and southern Taiwan; furthermore, the same analysis also indicated that the 3 main regions were isolated during the last Pleistocene glaciation (Wang et al. 2000). These phylogeographic patterns point out that the colonization, emigration, and migration of fish in Taiwan differ from the currently known routes. Therefore, in our study, a further phylogeographic analysis was performed to enhance our understanding of faunal formations of Taiwan.

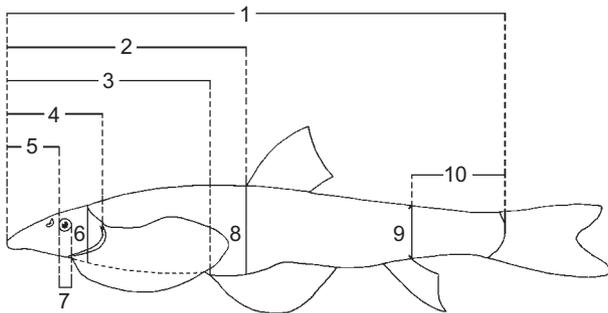
In contrast to previously studied fishes which swim well, the hillstream loach is a suitable model for studying evolutionary history of freshwater fishes because its native populations resulted from restrictions on hybridization by the various geographical barriers and river courses. In addition, hillstream loaches are less disturbed by human activities and have a lower economic value in comparison to other fishes. A phylogeographic analysis of endemic *Hemimyzon* of Taiwan is therefore likely to provide a more-accurate scenario of the influences of the CMR barrier on speciation, subdivision, and diversification of freshwater organisms. In this study, mitochondrial control region sequences were used as markers to construct the phylogeographic patterns of 2 species of *Hemimyzon*. A minimum spanning network and nested clade analysis revealed the spatial patterns and inferred the historical processes which may have led to the current distributions. In addition, genetic and morphological comparisons can further aid our understanding of the geo-historical influences of diversification at both the inter- and intraspecific levels.

## MATERIAL AND METHODS

### Sample collection and morphological measurements

Table 1 and figure 1 indicate the localities and details of specimens collected from 16 rivers in Taiwan. In total, 71 extracted DNA samples were sequenced, including 50 from *H. formosanus*, 18 from *H. taitungensis*, and 3 from *H. megalopseos* (which was used as the outgroup). The mitochondrial sequences of closely related genera were obtained from GenBank. Other outgroups utilized were *Jinshaia abbreviate* (AY600876), *J. sinensis* (DQ105282), and *Lepturichthys fimbriata* (DQ105283).

Eighteen morphological measurements and counts were obtained from each specimen of 59 samples. These samples consisted of 11 individuals of *H. taitungensis* and 48 individuals of *H. formosanus*. Measurements made with digital calipers were rounded up to the nearest 0.1 mm (Fig. 2).



**Fig. 2.** Measurements taken of *Hemimyzon* species. 1, standard length, measured from the tip of the head to the posterior edge of the hypural plate; 2, predorsal length, measured from the tip of the upper jaw to the anterior edge of the dorsal insertion; 3, prepelvic length, measured from the tip of the upper jaw to the anterior edge of the pelvic insertion; 4, head length, measured from the tip of the head to the posterior edge of the operculum; 5, snout length, measured from the tip of the head to the anterior margin of the osseous orbit; 6, head depth, at the level of the occiput; 7, orbital diameter, the distance between the horizontal margins of the osseous orbit; 8, body depth, at the level of the dorsal fin origin; 9, caudal peduncle depth, at the level of the end of the anal fin base; 10, caudal peduncle length, measured from the end of the anal fin base to the end of the hypural plate; the interorbital width is the cross distance between the upper margins of each osseous orbit; the head width is the cross distance between the anterior pectoral fin bases; the body width is the cross distance between the anterior pelvic fin bases; and the mouth width is the cross distance between the interior corners of the mouth.

### DNA extraction, polymerase chain reaction (PCR), and sequencing

A piece of pectoral fin or pelvic fin, of approximately 50 mg, was immersed in 500  $\mu$ l digestion buffer (10 mM Tris-HCl (pH 8), 1% SDS, 2 mM EDTA, 10 mM NaCl, 10 mg/ml DTT, and 0.5 mg/ml proteinase K; modified from Kocher et al. 1989); preparations were incubated for 16 h at 50°C in a dry bath. DNA was isolated and purified by a phenol/chloroform-isoamyl alcohol extraction (Innis et al. 1989). The control region of mtDNA was amplified and sequenced using the forward primers (PK2, PK3-1, and U2) and reverse primers (PU3-1 and PU2); the primers were designed in correspondence with the nucleotide positions in the light-chain of mtDNA of *Formosania lacustre* (M91245): PK3-1 (L68-L84, D-loop): 5'-TATTTA GACCATAAAGC-3'; U2 (L234-L253, D-loop): 5'-AGTAAGAAACCACCAACCAG-3'; PU3-1 (L912-L898, D-loop): 5'-TTAAGCTACGCTAGC-3'; PU2 (L1046-L1026, 12S-RNA): 5'-GGGCATTCT CACGGGGATGCG-3'; and PK2 (L164504-L16530, tRNA<sup>Thr</sup>): 5'-GTCGACTCTCACCCCTG GCTCCCAAAG-3'.

PCR amplifications were performed in a volume of 50  $\mu$ l containing 30-100 ng DNA, 200  $\mu$ M of each dNTP, 0.3  $\mu$ M of each primer, and 1 unit of *SuperTaq* with the reaction buffer (HT Biotechnology, Carbridge, UK). The PCR conditions were optimized as follows: a single hot start-up cycle of 93°C for 3 min; 35-40 cycles of denaturation at 93°C for 30 s, annealing at 40-55°C for 40 s, and extension at 72°C for 1 min; and a single final extension cycle of 72°C for 10 min. The amplification procedure was repeated 3 times. The product mixture was employed as the template for sequencing, which was performed using the Sequenase PCR Product Sequencing Kit (United States Biochemical, Illinois, USA) and dye-labeled terminator sequence kits (Applied Biosystems and Amersham Pharmacia, CA, USA) on an ABI model 377 automated DNA sequencer. Each haplotype was submitted to the GenBank database and assigned the following accession nos.: AY284892-AY284924 and AY541592-AY541597.

### Genetic divergence and phylogenetic analysis

Sequences were aligned using ClustalX (Thompson et al. 1997) and checked visually. Phylogenetic analyses were performed, first using the neighbor-joining (NJ) method (Saitou and Nei

1987) with Tamura-Nei (TN) gamma distances (Tamura and Nei 1993) and 5000 bootstrap replications (Felsenstein 1985), as implemented in MEGA (vers. 2.1, Kumar et al. 2001). Maximum-parsimony (MP) analyses were conducted using a random addition heuristic search with tree-bisection-reconnection (TBR), and 1000 bootstrap replications in PAUP\* (vers. 4.0b10, Swofford 1998). Genetic diversity was quantified at the inter- and intra-population levels using DnaSP (vers. 3.99, Rozas et al. 2003) to calculate the index of haplotype diversity ( $h$ ) (Nei 1987), estimates of nucleotide diversity ( $\pi$ ) (Nei 1987), and  $F_{ST}$  for gene flow (Hudson et al. 1992). A hierarchical analysis of molecular variance (AMOVA) was performed using Arlequin (vers. 2.0, Schneider et al. 2000) to compare the component of genetic diversity for the variance among subdivisions and species.

### Nested clade analysis (NCA)

Genetic data were also employed to establish a minimum spanning network with TCS vers. 1.13 (Clement et al. 2000), a tree that helps reveal information about distribution patterns (Chiang and Schaal 1999). The NCA combines genetic and geographical data to provide inferences about the recent geographic history of populations at the intraspecific level (Templeton 1998) and is performed using GeoDis (Posada et al. 2000). A nested structure derived from the minimum-spanning network, together with information on the geographical distribution of the haplotypes, is used to estimate 2 geographical measures for each clade: the clade distance (Dc) and the nested-clade distance (Dn). Dc is a measure of the geographical extent of a given clade, while Dn measures the average geographical distance of individuals from 1 clade to another in the next higher-level clade, within which it is contained. The analysis performed by GeoDis allows inference of a range of

**Table 1.** Sampling localities according to the zoogeographical zone, major basin, and population. Localities of the 2 species of *Hemimyzon* are followed by the number of individuals surveyed ( $n$ ), the number of unique haplotypes, mtDNA lineage, haplotype diversity ( $h$ ), and nucleotide diversity ( $\pi$ )

| Zoogeographical zone                     | Basin                    | Population | $n$ | Haplotypes     | mtDNA lineage | $h \pm SD$        | $\pi \pm SD$        |
|--|--------------------------|------------|-----|----------------|---------------|-------------------|---------------------|
| <i>H. formosanus</i> (Hf) <sup>c</sup>   |                          |            |     |                |               |                   |                     |
| Northern zone                            | Ilan River/Taiwan        | R1         | 2   | 2              | 2-2 (W1)      | 1.000 $\pm$ 0.500 | 0.0022 $\pm$ 0.0015 |
|  | Danshui River/Taiwan     | R2         | 6   | 5              | 2-2 (W1)      | 0.933 $\pm$ 0.122 | 0.0032 $\pm$ 0.0012 |
| Central/intermediate zone                | Touchien River/Taiwan    | R3         | 5   | 2              | 2-1 (W2)      | 0.400 $\pm$ 0.237 | 0.0009 $\pm$ 0.0006 |
|  | Zhongkong River/Taiwan   | R4         | 2   | 1 <sup>a</sup> | 2-1 (W2)      | 0.000 $\pm$ 0.000 | 0.0000 $\pm$ 0.0000 |
|  | Houlong River/Taiwan     | R5         | 3   | 2 <sup>a</sup> | 2-1 (W2)      | 0.667 $\pm$ 0.314 | 0.0007 $\pm$ 0.0008 |
|  | Taan River/Taiwan        | R6         | 6   | 4 <sup>a</sup> | 2-1 (W2,W3)   | 0.800 $\pm$ 0.172 | 0.0029 $\pm$ 0.0011 |
|  | Tachia River/Taiwan      | R7         | 3   | 2              | 2-1 (W3)      | 0.667 $\pm$ 0.314 | 0.0023 $\pm$ 0.0013 |
|  | Tadu River/Taiwan        | R8         | 5   | 1              | 2-1 (W3)      | 0.000 $\pm$ 0.000 | 0.0000 $\pm$ 0.0000 |
|  | Choshui River/Taiwan     | R9         | 6   | 1              | 2-1 (W3)      | 0.000 $\pm$ 0.000 | 0.0000 $\pm$ 0.0000 |
| Southern zone                            | Tzengwen River/Taiwan    | R10        | 7   | 3              | 3-2 (W4)      | 0.524 $\pm$ 0.209 | 0.0012 $\pm$ 0.0007 |
|  | Kaoping River/Taiwan     | R11        | 5   | 2              | 3-3 (W5)      | 0.400 $\pm$ 0.237 | 0.0005 $\pm$ 0.0005 |
| <i>H. taitungensis</i> (Ht) <sup>d</sup> |                          |            |     |                |               |                   |                     |
| Eastern zone                             | Taimali River/Taiwan     | R12        | 2   | 1              | 2-4 (E2)      | 0.000 $\pm$ 0.000 | 0.0000 $\pm$ 0.0000 |
|  | Zhiben River/Taiwan      | R13        | 2   | 1              | 2-4 (E2)      | 0.000 $\pm$ 0.000 | 0.0000 $\pm$ 0.0000 |
|  | Peinan River/Taiwan      | R14        | 4   | 4              | 2-3 (E1)      | 1.000 $\pm$ 0.177 | 0.0047 $\pm$ 0.0016 |
|  | Hsiukuluang River/Taiwan | R15        | 2   | 2 <sup>b</sup> | 2-3 (E1)      | 1.000 $\pm$ 0.500 | 0.0011 $\pm$ 0.0011 |
|  | Hualien River/Taiwan     | R16        | 8   | 4 <sup>b</sup> | 2-3 (E1)      | 0.643 $\pm$ 0.184 | 0.0014 $\pm$ 0.0006 |
|  |                          |            |     |                |               |                   |                     |
| <i>H. megalopseos</i> (Hm)               | Nanpan River/China       | Hm         | 3   | 2              | -             | 0.667 $\pm$ 0.314 | 0.0082 $\pm$ 0.0023 |

<sup>a</sup>Three populations share a common Hf8 haplotype. <sup>b</sup>Two populations share a common Ht8 haplotype. <sup>c</sup>The average haplotype diversity and nucleotide diversity of all pooled samples within the species were 0.495  $\pm$  0.013 and 0.0149  $\pm$  0.0022, respectively. <sup>d</sup>The average haplotype diversity and nucleotide diversity of all pooled samples within the species were 0.889  $\pm$  0.064 and 0.0049  $\pm$  0.0010, respectively.

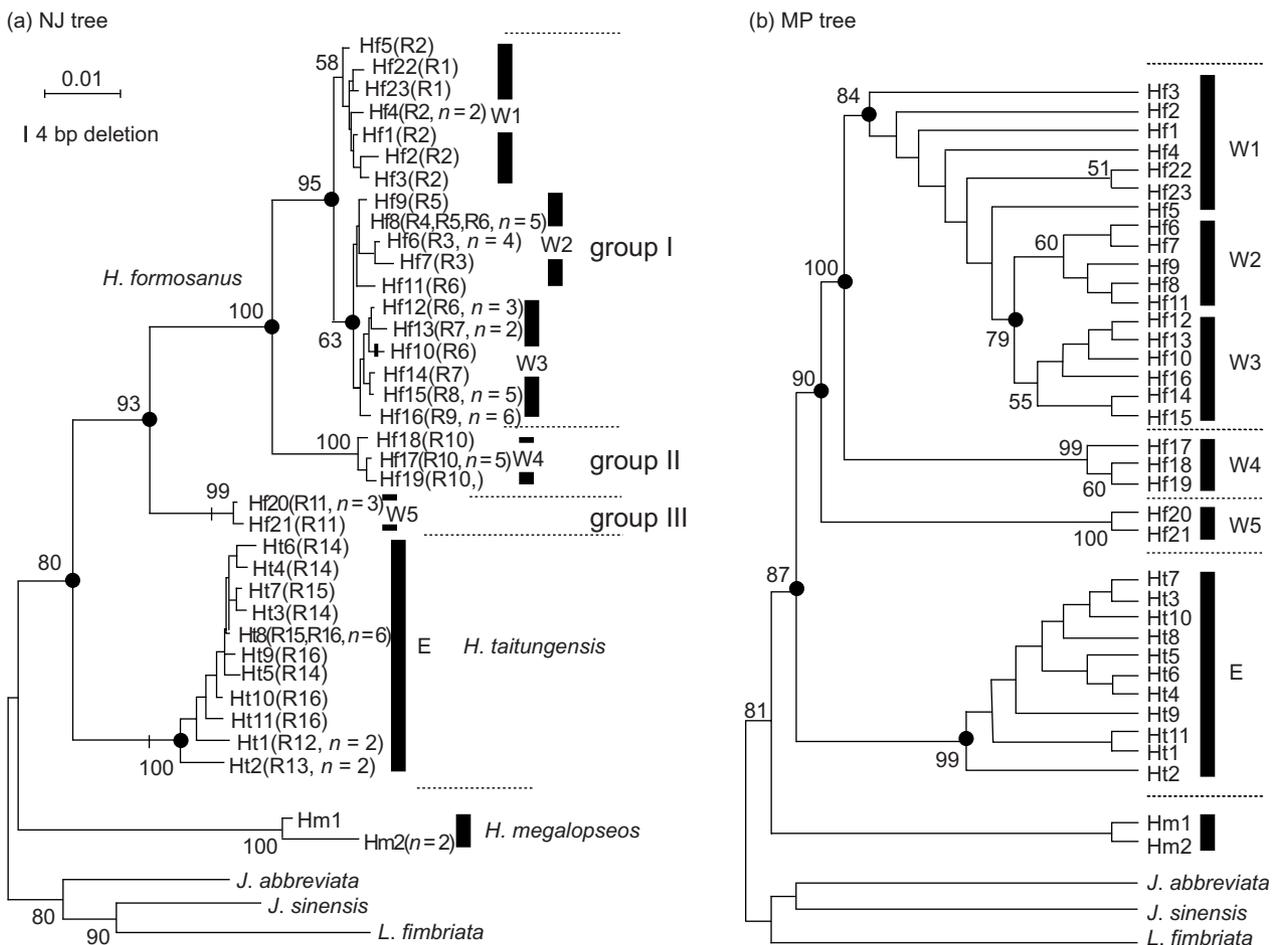
phylogeographic processes, including range expansion (either contiguously or by long-distance colonization), isolation by distance due to restricted gene flow, fragmentation of populations (including extinction of intermediate populations), and various combinations of these possibilities. There are 4 steps in the NCA: constructing a haplotype network, nesting clades on the network, testing for geographic associations, and determining inferences about the processes that have generated the pattern. An inference key was provided by Posada and Templeton (2005), and updates (the most recent of which was used here) are available on the GeoDis website.

## RESULTS

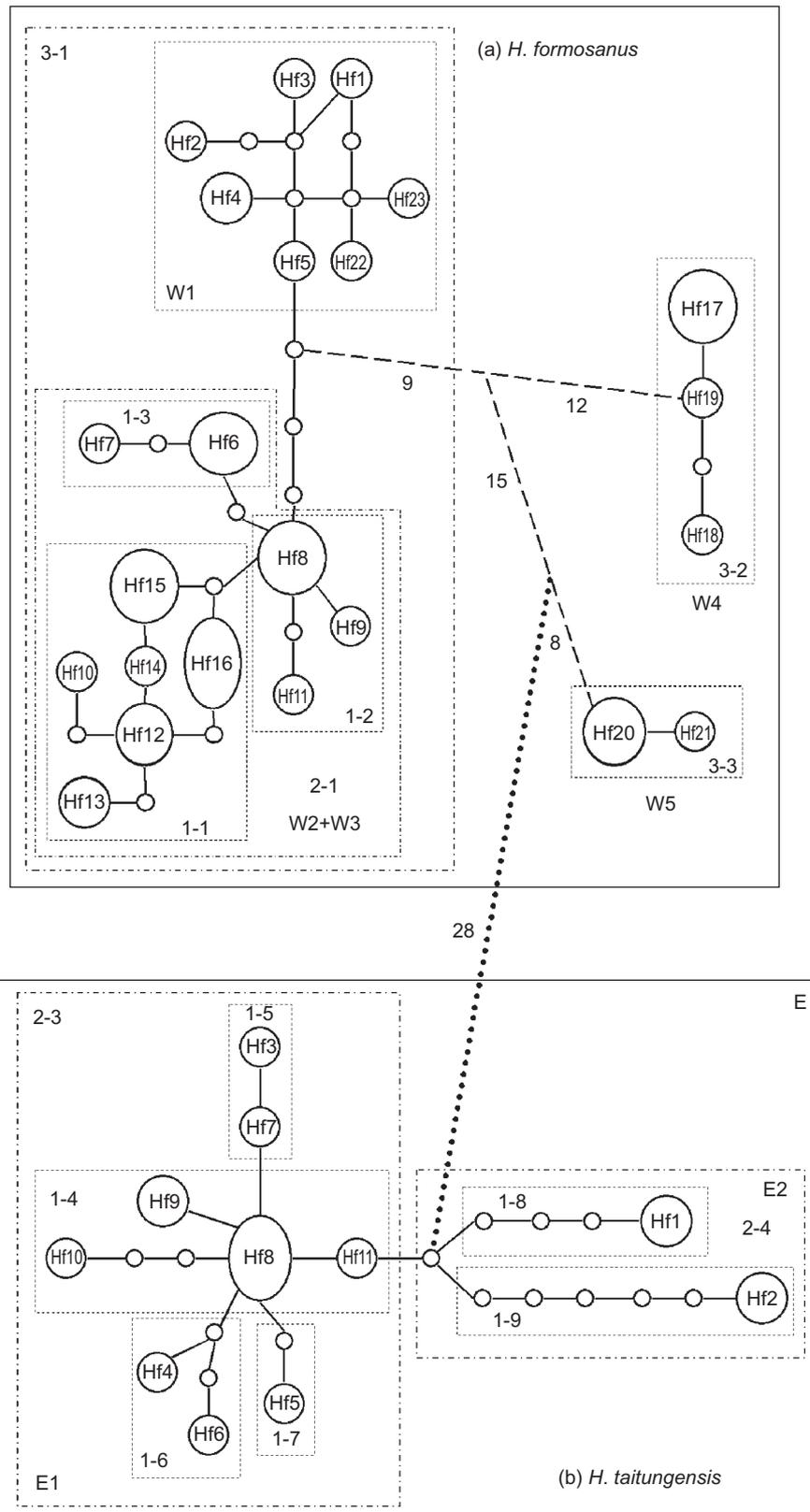
### Mitochondrial DNA variations

In both *H. formosanus* and *H. taitungensis*, the length of the D-loop regions within mitochondria ranged from 893 to 899 base pairs (bp). In addition, the average nucleotide compositions in the D-loop region were 31.8% T, 18.3% C, 36.0% A, and 13.9% G. Furthermore, the nucleotide compositions indicated that this region within both species was AT-rich, and similar observations were found in many other vertebrates (Brown et al. 1986, Tzeng et al. 1992, Lee et al. 1995, Perdices and Doadrio 2001).

In *H. formosanus*, 23 haplotypes were identi-



**Fig. 3.** Phylogenetic tree of the 2 balitorid fishes. (a) Neighbor-joining (NJ) phylogram constructed from Tamura-Nei (gamma) distances in the D-loop region with 3 major groups in *Hemimyzon formosanus*. (b) Three groups within the maximum-parsimony (MP) cladogram. The numbered nodes indicate NJ/MP bootstrap values (%). Values for each subgroup are shown. Abbreviations are explained in table 1.



**Fig. 4.** Minimum spanning haplotype network. (a) In *Hemimyzon formosanus*; (b) in *H. taitungensis*. Unfilled circles indicate unsampled intermediate haplotypes with a single mutation from the neighboring haplotype. Numbers next to the dotted and dashed lines indicate the numbers of mutational events which occurred.

fied, most of which were not shared among different populations (Table 1). However, 1 haplotype (Hf8) was shared in populations from 3 rivers: the Zhongkong (R4), Houlong (R5), and Taan (R6) Rivers. On the other hand, in *H. taitungensis*, 11 haplotypes were discovered, and 1 shared haplotype (Ht8) existed in populations from the Hsiukuluan (R15) and Hualien (R16) Rivers.

A 4-bp indel located at the 5'-end of the control region in mtDNA was identified in populations of both species. The indel was observed in all haplotypes of the Kaoping River (R11) in *H. formosanus* (Fig. 3a), and was present in all haplotypes of *H. taitungensis*.

Among *H. formosanus*, the average haplotype diversity ( $h$ ) of samples from all populations was  $0.495 \pm 0.013$ , and the average nucleotide diversity ( $\pi$ ) of samples from all populations was  $0.0149 \pm 0.0022$  (Table 1). On the other hand, in *H. taitungensis*, the average haplotype diversity of samples from all population was  $0.889 \pm 0.064$ , and the average nucleotide diversity of samples from all populations was  $0.0049 \pm 0.0010$ . By comparison, the average haplotype diversity was about 2 times higher in *H. taitungensis* than in *H. formosanus*. The average nucleotide diversity was 3 times higher in *H. formosanus* than in *H. taitungensis*.

### Phylogenetic patterns

Populations of *H. formosanus* were divided into 3 major groups: group I consisted of populations W1, W2, and W3; group II contained population W4, and group III consisted of population W5 (Fig. 3a). The phylogenetic relationships between each group were supported by high bootstrap values and by high confidence from the interior branch test (Nei et al. 1985, Nei and Kumar 2000) (data not shown). In addition, the 3 major groups diverged at different genetic distances. A similar pattern of evolutionary relationships was also observed in the MP tree (Fig. 3b). Although the W1 group might have appeared paraphyletic in the MP tree, it appeared monophyletic in the NJ tree (with a bootstrap value of 95) and in the haplotype network (with 3 mutational events). Therefore, W1 could be classified as a subgroup of group I.

Populations from central Taiwan (W2 and W3) were shown to be a hybrid group, which could be separated into 2 subgroups with intermediate bootstrap values (of 63 and 79) in both the NJ and MP trees. The slightly lower bootstrap values were

because the haplotypes from the Taan River were classified into both groups W2 and W3. Group W2 included populations from the Touchien (R3), Zhongkong (R4) and Houlong (R5) Rivers, and 1 individual from the Taan River (R6). On the other hand, group W3 consisted of populations from the Tachia (R7), Tadu (R8), and Choshui (R9) Rivers, and 4 individuals from the Taan River (R6). Although AMOVA indicated genetic structural differences within the subgroups (Table 2), populations from W2 and W3 were indiscernible and were classified into a single group.

From both the NJ and MP trees of *H. taitungensis*, a monophyletic group (E) with high bootstrap values (100 and 99) was comprised of all haplotypes with a short branch length, and all following subdivisions revealed low bootstrap values, implying no further subdivisions, unlike the deep genetic divergence among groups I, II, and III of *H. formosanus*.

### Haplotype networks and the NCA

The haplotype networks were generally congruent with the NJ and MP trees (Fig. 4). Eleven populations of *H. formosanus* were divided into 3 major groups (clades 3-1, 3-2, and 3-3) in the haplotype network. Greater mutational events were discovered among clades 3-1 (groups W1+W2+W3), 3-2 (group W4), and 3-3 (group W5). Furthermore, clade 3-1 contained clades 2-1 and 2-2; clade 2-1 consisted of groups W2 (clades 1-2 and 1-3) and W3 (clade 1-1), and clade 2-2 included group W1. Although W3 formed a subgroup, groups W2 and W3 merged in the haplotype network due to the shared haplotypes from the Taan River (Hf8, Hf10, Hf11, and Hf12). Both the phylogenetic analysis and haplotype network classified the 11 populations of *H. formosanus* into 3 major groups and 2 subgroups.

However, in *H. taitungensis*, results from the constructed NJ and MP trees were inconsistent with the results from the haplotype network because 2 subgroups (clades 2-3 and 2-4) were identified in the latter method. Clade 2-3 (E1) represented populations from 3 east-central rivers, while clade 2-4 (E2) represented 2 southeastern populations (from the Taimali and Zhiben Rivers). This inconsistency could be explained by the enhanced sensitivity of the haplotype network (Templeton 1998); moreover, in the "Discussion" section, the haplotype network results were given greater emphasis over the NJ and MP tree results.

The most recent NCA key (Posada and Templeton 2005) was used to infer the most likely geographic patterns and their associations in the evolutionary history (Table 3). The information provided by the NCA key indicated that the 3 major divisions were caused by past fragmentation in *H. formosanus*, and long-distance colonization/range expansion occurred in *H. taitungensis*.

### Genetic differentiation

Deep genetic divergence was observed between the Kaoping population (W5) and the remaining populations of *H. formosanus*. The genetic distance within group W5 ( $0.05\% \pm 0.05\%$ ) was the lowest, while group W1 had the highest within-group distance ( $0.35\% \pm 0.12\%$ ; Table 4). The genetic distance between groups was the highest between groups W3 and W5 ( $4.09\% \pm 0.65\%$ ), and was the lowest between groups W2 and W3 ( $0.41\% \pm 0.13\%$ ).

In *H. taitungensis*, the genetic distance within the southern group E2 ( $0.77\% \pm 0.25\%$ ) was 3 times higher than in the central group E1 ( $0.24\% \pm 0.07\%$ ). In addition, the genetic distance between these 2 groups was  $0.91\% \pm 0.25\%$ , which is slightly higher than the genetic distance within group E2, and it was much higher than the distance within group E1 (Table 4).

The interspecific genetic distance was  $3.99\% \pm 0.65\%$  between the Kaoping population (W5) of *H. formosanus* and the 2 southeastern populations

(E2) of *H. taitungensis*. This genetic distance was almost equal to the value between the Kaoping population and the remaining populations of *H. formosanus* ( $3.91\% \pm 0.66\%$  to  $4.09\% \pm 0.65\%$ ; mean value,  $4.03\% \pm 0.64\%$ ), but was 1/3 lower than the mean distance between the 2 species (mean value,  $5.87\% \pm 0.81\%$ ).

The high  $F_{ST}$  values indicated the occurrence of low gene flow between the groups (except for *H. taitungensis* and group I of *H. formosanus*; Table 4). A moderate  $F_{ST}$  value was calculated between groups E1 and E2, and slightly higher  $F_{ST}$  values were calculated between groups W1 and W2 and between groups W1 and W3. In addition, a higher frequency of gene flow appeared between groups W2 and W3 in west-central Taiwan.

### Morphological comparisons

Due to the deep genetic divergence ( $4.03\%$ ) of the southern population of *H. formosanus*, it is possible to classify the Kaoping population as a new biological or phylogenetic species or subspecies. Morphological comparisons can help explain this variance in genetic diversity. Forty-eight specimens of *H. formosanus* and 11 *H. taitungensis* specimens were morphometrically analyzed. In total, 18 measurements and counts were made (Table 5). According to the biogeographic analysis described by Tzeng (1986), we separated *H. formosanus* ranges into 3 main zones (northern, southern, and central/intermedi-

**Table 2.** Hierarchical analysis based on the genetic distance among species, subdivisions and distinct zones of *Hemimyzon formosanus*

| Source of variation                      | d.f. | Sum of squares | Variance components | Percent of variation |
|--|------|----------------|---------------------|----------------------|
| Among species                            | 1    | 589.632        | 19.26579            | 70.77*               |
| Among subdivisions within species        | 5    | 299.877        | 6.83732             | 25.11**              |
| Within subdivisions                      | 60   | 67.302         | 1.12169             | 4.12**               |
| Total                                    | 66   | 956.811        | 27.22480            |                      |
| Among subdivisions                       | 4    | 275.881        | 7.72604             | 84.63**              |
| Among populations within subdivisions    | 5    | 19.020         | 0.90929             | 9.96**               |
| Within populations                       | 33   | 16.310         | 0.49424             | 5.41**               |
| Total                                    | 42   | 311.211        | 9.12956             |                      |
| Among distinct zones                     | 2    | 179.709        | 3.17979             | 33.50                |
| Among subdivisions within distinct zones | 2    | 105.851        | 5.32995             | 56.15**              |
| Within subdivisions                      | 44   | 43.240         | 0.98273             | 10.35**              |
| Total                                    | 48   | 328.801        | 9.49248             |                      |

\*  $p < 0.05$ ; \*\*  $p < 0.01$ .

ate zones). The northern zone was comprised of group W1; the central zone included groups W2 and W3; while groups W4 and W5 belonged to the southern zone. Unfortunately, none of these measurements could be utilized to establish group W5 as a new species due to large morphological variations within the central group. However, after the specimens were excluded from the central/intermediate zone, 2 measurements were determined that can help discern specimens between the northern and southern zones. The 2 measurements are the values of the lateral line scales and the ratios of the standard length to the caudal peduncle depth. According to the ANOVA test, some groups exhibited a significant difference in these 2 measurements.

## DISCUSSION

The phylogeographical and morphological analyses of *H. formosanus* and *H. taitungensis* offer a new scenario for the speciation of aquatic

organisms in Taiwan. Our data suggest that the population of *H. formosanus* in western Taiwan can be divided into 3 groups and 2 subgroups. This division pattern is partially congruent with the patterns of other freshwater fishes. In addition, mean genetic distances revealed that the Kaoping population of *H. formosanus* (group W5) is more divergent from the other *H. formosanus* populations than from *H. taitungensis*. These results indicate the presence of cryptic species in the Kaoping River and an unusual evolutionary history for these 2 balitorids in Taiwan.

## Phylogeographical implications

The phylogenetic tree and NCA inferences present a scenario of the evolutionary history of *H. formosanus* in Taiwan: the spatial patterns are the results of 3 major fragmentations followed by subsequent local range expansion and colonization. The 3 major groups (W1+W2+W3, W4, and W5) of *H. formosanus* are potentially consequences of past fragmentations (Table 3). The genetic differ-

**Table 3.** Chains of inference from the nested clade analysis. Haplotype and clade designations are given in figure 4

| Clade                       | Inference chain       | Inferred pattern   |
|-----------------------------|-----------------------|--|
| <i>Hemimyzon formosanus</i> |                       |  |
| 1-1                         | 1-2-3-5-6-13-14-15 NO | Past fragmentation (PF) or long-distance colonization (LDC)  |
| 2-1                         | 1-2-11-12-13-14 NO    | Colonization event is inferred, perhaps associated with recent fragmentation (CRF)   |
| 3-1                         | 1-2-11-12-13 YES      | Past fragmentation followed by range expansion (PF-RE)   |
| Total                       | 1-2-11-12-13-14 NO    | Past fragmentation (PF)  |
| <i>H. taitungensis</i>      |                       |  |
| 2-3                         | 1-2-3-4 NO            | Restricted gene flow with isolation by distance (RGF)  |
| Total                       | 1-2-11-12-13 YES      | Long-distance colonization possibly coupled with subsequent fragmentation (LDC-SF) or past fragmentation followed by range expansion (PF-RE) |

**Table 4.** Genetic divergence and standard deviation (%) within (bold) and between (lower left matrix) different subdivisions; the upper right matrix indicates the  $F_{ST}$  value. High  $F_{ST}$  values (0.8-1.0) reveal low gene flow between subdivisions

|    | W1          | W2                 | W3                 | W4          | W5          | E2          | E1                 |
|----|-------------|--------------------|--------------------|-------------|-------------|-------------|--------------------|
| W1 | 0.35 ± 0.12 | 0.650 <sup>a</sup> | 0.618 <sup>a</sup> | 0.878       | 0.956       | 0.939       | 0.908              |
| W2 | 0.81 ± 0.25 | 0.18 ± 0.09        | 0.455 <sup>a</sup> | 0.925       | 0.977       | 0.957       | 0.926              |
| W3 | 0.94 ± 0.25 | 0.41 ± 0.13        | 0.26 ± 0.09        | 0.883       | 0.950       | 0.936       | 0.907              |
| W4 | 2.32 ± 0.49 | 2.61 ± 0.54        | 2.65 ± 0.54        | 0.12 ± 0.07 | 0.971       | 0.955       | 0.925              |
| W5 | 3.91 ± 0.66 | 4.01 ± 0.67        | 4.09 ± 0.65        | 4.03 ± 0.67 | 0.05 ± 0.05 | 0.961       | 0.918              |
| E2 | 5.71 ± 0.85 | 5.87 ± 0.86        | 6.06 ± 0.87        | 6.21 ± 0.92 | 3.99 ± 0.65 | 0.77 ± 0.25 | 0.512 <sup>a</sup> |
| E1 | 5.77 ± 0.87 | 5.94 ± 0.88        | 6.12 ± 0.90        | 6.36 ± 0.96 | 4.47 ± 0.70 | 0.91 ± 0.25 | 0.24 ± 0.07        |

<sup>a</sup>Moderate  $F_{ST}$  values (0.3-0.7); low  $F_{ST}$  values (0-0.2).

entiation within the species of *H. formosanus* is 2-3 times greater than those seen in *A. paradoxus* and *V. barbatulus*. The different degrees of divergence, along with the regional separations deduced by NCA inferences, imply the occurrence of early fragmentations. Furthermore, the molecular clock calculations suggest that fragmentations appeared during the early Pleistocene (Wang et al. 2007). Therefore, this deep divergence may have been due to earlier subdivisions related to isolated refugia, lower gene flow, or specific habits separating populations of *H. formosanus* from north-cen-

tral and southern Taiwan.

On the other hand, recent fragmentations and local range expansion took place in central Taiwan. Herein, the inference of nested clade 3-1 suggests that the separation of the northern and central groups likely resulted from recent fragmentation followed by range expansion. In addition, clade 2-1 (W2+W3) was inferred to have resulted from a colonization event followed by recent fragmentation in central Taiwan. During the middle or late Pleistocene, a decrease in the sea level or flooding in central Taiwan resulted in changes in the river

**Table 5.** Morphological comparison of *Hemimyzon formosanus* and *H. taitungensis* (units: mm). Unbranched fin-rays are represented as Roman numerals and branched rays are represented as Arabic numerals. *n* is the number of individuals sampled; *p* is the number of populations within each area. Bold and underlined letters indicate differences between the 2 species and 2 subdivisions, respectively

| Distinct zone                                  | <i>H. formosanus</i>      |                           |                    |                          |                             | <i>H. taitungensis</i> |
|--|---------------------------|---------------------------|--------------------|--------------------------|-----------------------------|------------------------|
|  | Northern zone             | Central/intermediate zone |                    |                          | Southern zone               |                        |
| Subdivision                                    | W1                        | W2                        | W3                 | W4                       | W5                          | E1+E2                  |
| <i>n</i> ( <i>p</i> )                          | 4 (1)                     | 6 (2)                     | 25 (4)             | 8 (1)                    | 5 (2)                       | 11 (3)                 |
| Dorsal fin rays                                | iii-8                     | iii-8                     | iii-8              | iii-8                    | iii-8                       | iii-8                  |
| Anal fin rays                                  | ii-5                      | ii-5                      | ii-5               | ii-5                     | ii-5                        | ii-5                   |
| Pectoral fin rays                              | x-xi/10-11                | x-xii/9-11                | x-xii/8-11         | ix-xii/10-11             | xii/10-11                   | <b>xii-xv/10-13</b>    |
| Ventral fin rays                               | iv/9                      | iv/7-10                   | iii-v/8-11         | iii-iv/8-9               | iv-v/9-10                   | <b>v-vii/10-11</b>     |
| Lateral line scales                            | <u>68-71 (70)**</u>       | 70-76 (74)                | 68-75 (71)**       | <u>72-76 (74)**</u>      | <u>70-73 (72)</u>           | <b>78-81 (79)</b>      |
| Standard length                                | 32.0-59.4 (43.0)          | 58.6-74.0 (65.0)          | 37.6-73.5 (52.07)  | 45.1-60.0 (52.9)         | 34.7-49.1 (51.7)            | 49.9-73.0 (61.0)       |
| Standard length / body depth                   | 6.67-7.96 (7.43)          | 5.95-7.89 (6.91)          | 5.48-9.09(7.02)    | 7.27-8.34 (7.65)         | 7.17-8.45 (7.92)            | 6.27-8.80 (7.50)       |
| Standard length / body width                   | 4.18-4.83 (4.54)          | 3.81-4.56 (4.29)          | 3.57-5.40(4.15)    | 4.22-4.97 (4.72)         | 3.99-4.75 (4.47)            | 4.54-5.29 (4.89)       |
| Standard length / head length                  | 4.95-5.33 (5.17)          | 4.61-5.83 (5.04)          | 4.33-5.69(4.86)    | 4.74-5.61 (5.11)         | 4.47-5.27 (4.86)            | 4.70-5.37 (5.01)       |
| Standard length / caudal peduncle length       | 6.15-7.66 (6.95)          | 6.81-8.06 (7.46)          | 6.22-9.41(7.41)    | 6.99-7.87 (7.58)         | 6.33-8.00 (7.22)            | 6.16-7.23 (6.57)       |
| Standard length / caudal peduncle depth        | <u>8.29-8.89 (8.61)**</u> | 9.11-10.57 (9.85)         | 8.05-11.79(10.1)** | <u>9.40-10.76 (10.2)</u> | <u>10.52-12.93 (11.5)**</u> | 9.16-10.85 (9.76)      |
| Head length / head depth                       | 1.62-1.74 (1.68)          | 1.74-1.94 (1.86)          | 1.63-2.01(1.83)    | 1.69-1.93 (1.79)         | 1.73-2.31 (2.02)            | 1.77-2.26 (1.97)       |
| Head length / head width                       | 0.87-0.97 (0.92)          | 0.91-1.05 (0.97)          | 0.81-1.05(0.93)    | 0.85-1.04 (0.93)         | 0.89-1.05 (0.98)            | 0.94-1.13 (1.00)       |
| Head length / snout length                     | 2.13-2.17 (2.15)          | 1.90-2.30 (2.06)          | 1.84-2.23(2.04)    | 1.88-2.25 (2.05)         | 1.97-2.07 (2.02)            | 1.93-2.51 (2.18)       |
| Head length / orbital diameter                 | 4.62-5.23 (4.93)          | 5.08-6.00 (5.56)          | 4.11-6.45(5.49)    | 5.00-6.33 (5.63)         | 4.72-5.47 (5.00)            | 5.19-7.76 (6.02)       |
| Head length / interorbital width               | 1.88-2.21 (2.04)          | 1.76-2.31 (1.98)          | 1.58-2.15(1.85)    | 1.67-2.18 (1.86)         | 1.81-2.07 (1.97)            | 2.11-2.41 (2.22)       |
| Caudal peduncle length / caudal peduncle depth | 1.13-1.44 (1.25)          | 1.14-1.54 (1.33)          | 1.05-1.85(1.38)    | 1.21-1.51 (1.35)         | 1.51-1.71 (1.59)            | 1.34-1.76 (1.49)       |
| Head width / mouth width                       | 2.74-3.00 (2.88)          | 2.51-2.93 (2.73)          | 2.42-3.28(2.87)    | 2.50-2.84 (2.66)         | 2.52-2.88 (2.70)            | 2.41-3.20 (2.85)       |

\*\* *p* < 0.01 by one-way ANOVA with each other

systems (Lin 1957, Emery et al. 1971); thus the hillstream loach populations may have dispersed and interacted with each other in central Taiwan. However, owing to the increase in the sea level or the appearance of geographic boundaries, the populations may have been re-isolated from one to another. Alternate dispersal and vicariance events may have been the driving forces for interactions of groups W2 and W3; therefore, slightly diversified subgroups still exist as supported by the NJ tree. This explanation is supported by the identification of a shared haplotype (Hf8) and moderate gene flow ( $F_{ST} = 0.455$ ) between the 2 populations in the central region. Similar inferences were discovered in the analyses of *A. paradoxus* and *V. barbatulus* in central Taiwan as well.

The haplotype network divided the populations of *H. taitungensis* into 2 nested clades (Fig. 4). The causes of the separation of clades 2-3 (group E1) and 2-4 (group E2) remain uncertain. Two possible causes are proposed: the separation could have resulted from long-distance colonization coupled with subsequent fragmentation, or from recent fragmentation followed by 1 or more range expansions. Group E1, which includes most of the haplotypes and was constructed as a lower-level clade, was inferred to have restricted gene flow due to isolation by distance (Table 3). As suggested by the center of the haplotype network (clade 1-4), the Hualien River (R16) could be the expansion center for *H. taitungensis* in eastern Taiwan. In our study, a long geographical distance between the expansion center and group E2 was observed. In addition, the shared haplotype (Hf8) and moderate  $F_{ST}$  value (0.512) between groups E1 and E2, evidence for the occurrence of gene flow, imply that at least 1 dispersal event took place in eastern Taiwan. Therefore, long-distance colonization coupled with subsequent fragmentation appears to be a better explanation for the results of the haplotype network. A low genetic distance (< 1%) and moderate gene flow indicate at least 1 recent colonization event in eastern Taiwan, which is similar to the pattern observed for *V. barbatulus* during the last Pleistocene glaciation.

The molecular clock estimates of the *cytochrome b* gene revealed that the speciation and sequential subdivisions of *Hemimyzon* species occurred approximately 2-4 million yr ago (Wang et al. 2007). The speciation time of the 2 species is in the vicinity of the formation time of the CMR barrier, which implies that the CMR barrier may

have participated in separating the common ancestor of *H. formosanus* and *H. taitungensis* into their present distributions in western and eastern Taiwan. Sequential subdivisions in *H. formosanus* may have played crucial roles in the genetic variations in northern, central, and southern populations, the strength of which is correlated to different divergence times and degrees of isolation in the west.

### Genetic and morphological implications

The deep genetic divergence implies that cryptic species might have arisen from the southern population of *H. formosanus*, which is unique in differing from the shallow genetic divergence known for other freshwater fishes in Taiwan. The AMOVA indicated significant spatial patterns of genetic structure among each group within the distinct zones (Table 2). Both the phylogenetic tree and NCA supported the divergence patterns of each group. Furthermore, the results indicated slight morphological differences between the northern and southern groups (Table 5). A recent study (Chen and Chang 2005) also described several morphological differences between the southern population of *H. formosanus* and other populations, which supports the southern population being a cryptic species. Both measurements could be utilized to determine the possibility of the cryptic species being classified as a distinct species from *H. formosanus*.

From our study, a 4-bp indel was identified in 1 cryptic species from the Kaoping population (W5) and all populations of *H. taitungensis*. However, this indel was absent from the northern population (W1) and most of the central populations (W2 and W3) of *H. formosanus*, except for 1 haplotype in the Taan River. Thus the cryptic species exhibited a higher degree of similarity to *H. taitungensis* than to *H. formosanus*. Moreover, the genetic distance ( $3.99\% \pm 0.65\%$ ) between the Kaoping population (W5) of *H. formosanus* and southeastern population (E2) of *H. taitungensis* was equal to the mean distance ( $4.03\% \pm 0.64\%$ ) between the W5 group and other populations of *H. formosanus*. The deep genetic distance between the Kaoping population and other populations of *H. formosanus* implies a new phylogenetic species in southern Taiwan. The phylogeographic analysis, the variance in the morphology/mitochondrial sequence length, the deep genetic distance, and

the isolated habitats all suggest that the Kaoping population of *H. formosanus* may have resulted from early fragmentation, and it may correspond to a new cryptic species.

In conclusion, the phylogeography reveals that during the Pleistocene, the main factor separating populations of *H. formosanus* was sequential fragmentations, and the main cause for the emergence of *H. taitungensis* was long-distance colonization. Genetic and morphological variations imply that the Kaoping population of *H. formosanus* represents a phylogenetic species or subspecies. Future studies should focus on detailed analyses of anatomical comparisons and reproductive barriers to confirm the identity of the cryptic species.

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