Mitochondrial phylogeny reveals the artificial introduction of the pale chub *Zacco platypus* (Cyprinidae) in Taiwan

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Abstract The presence of the pale chub *Zacco platypus* (Japanese name, *Oikawa*) in Taiwan has been suggested to be a result of its inadvertent introduction from Lake Biwa in Japan in the 1980s in conjunction with the Japanese Ayu, *Plecoglossus altivelis altivelis*, which was released several times into the Tamsui River to restock the extinct Ayu population of Taiwan. However, it is also possible that *Z. platypus* is native to Taiwan and has not been previously described for reasons of its narrow range. Knowledge of the colonizing history of *Z. platypus* is of considerable importance because it provides insight into the evolutionary process and, hence, impacts management decisions regarding this species in Taiwan. A portion of the mitochondrial D-loop was sequenced for 77 specimens from five populations of *Z. platypus* from Japan and Taiwan. A total of 22 haplotypes were identified, and nucleotide divergence among haplotypes ranged from 0.20% to 2.82%. Haplotype diversity was high in all populations examined, with a range from 0.718 in the Tagiri River population to 0.909 in the Lake Biwa population. Phylogenetic and statistical parsimony analyses of the molecular data revealed a close genetic relationship between Taiwanese and Japanese *Z. platypus* and supported the previous report that the Taiwanese *Z. platypus* originated in Lake Biwa in Japan.

Key words *Zacco platypus* · Pale chub · Taiwan · Introduced species · Mitochondrial DNA

The pale chub *Zacco platypus* (Cyprinidae) has been long considered a single species across the whole of East Asia (Bănărescu, 1968; Chen 1982; Shen et al., 1993). However, this species was reported as comprising more than one taxon (i.e., a de facto species complex) because there is considerable genetic differentiation among populations (Ming, 1991; Perdices et al., 2004; Berrebi et al., 2005). Recently, two morphologically and genetically distinct species have been recognized in the pale chub (Ma et al., 2006): *Zacco platypus* (Japanese name, *Oikawa*), which is distributed in Japan, Korea, and Taiwan, and *Zacco* sp., which is found in China and Taiwan. In Taiwan, both species are restricted to the northern region of the island, but the distribution of *Z. platypus* is narrower than that of *Zacco* sp.; *Z. platypus* is only found in two tributaries of the Tamsui drainage (Keelung River and Hsintien River), whereas *Zacco* sp. occurs across the whole northern area (Ma et al., 2006). *Zacco* sp. is morphologically identical with the previous recorded "Formosan pale chub," *Zacco evolans* (also see Tzeng, 1986; Shen et al., 1993) and, therefore, it has been considered to be native to Taiwan (Chen and Chang, 2005; referring to this species as *Opariichthys evolans*). Consequently, we tentatively conclude that *Zacco evolans* is distributed in Taiwan and China.

The origin of *Z. platypus* in Taiwan is uncertain. However, undocumented anglers' reports on its occurrence are known, dating back less than 20 years. Chen and Chang (2005) argued in favor of a recent introduction of this species, probably through inadvertent releases in conjunction with the Japanese Ayu, *Plecoglossus altivelis altivelis*, which was restocked several times into the Tamsui River in the 1980s from Lake Biwa, Japan (Liu, 1995). Alternatively, it is also possible that *Z. platypus* is native to Taiwan and has never been noticed over the years because of its narrow range. Knowledge of the colonizing history of *Z. platypus* is of considerable importance because it provides insight into the evolutionary process and, hence, impacts management decisions of this species in Taiwan. Molecular genetic markers offer a means of determining the origin of this species. Because Taiwan and Japan have been completely isolated from each other by sea for more than 1 million years (Kimura, 2000), substantial genetic differentiation between Taiwanese and Japanese samples should be detectable if *Z. platypus* is native to Taiwan. Alternatively, the genetic differentiation between Taiwanese and Japanese samples is expected to be small and negligible if Taiwanese *Z. platypus* was a recent introduction from Japan.

The present study examines the genetic differentiations between *Z. platypus* from Taiwan and from Japan and aims to understand the colonizing history of *Z. platypus* in Taiwan. We obtained partial mitochondrial D-loop DNA sequences (mtDNA D-loop) from Japanese and Taiwanese fish. Phylogenetic and statistical parsimony analyses of the molecular data describe the genetic relationships and the origin of Taiwanese *Z. platypus*.

Materials and Methods

Sampling and DNA extraction.—Two groups of the pale chub *Zacco platypus* ($n = 77$) were analyzed. The first group consisted of fish from Taiwan, where the occurrence of *Z. platypus* has been suggested to be the result of species introduction from Japan (Chen and Chang, 2005). In total, 45 fish were collected from two tributaries of the Tamsui drainage (Keelung River, $n = 13$ and Hsintien River, $n = 32$), comprising sampling locations across the whole range of the species distribution in Taiwan (Fig. 1). The second group consisted of fish from Japan, where the pale chub is distributed from middle to southern regions of the country (Nakabo, 1993). Thirty-two fish were collected from four rivers in Japan (Fig. 1): (1) a small creek near Lake Biwa ($n = 8$), Moriyama, Shiga Prefecture; (2) Fugenji River ($n = 3$), Kyotanabe, Kyoto Prefecture; (3) Tagiri River ($n = 13$), Inabe River system, Mie Prefecture; and (4) Ara River ($n = 8$), Tokyo. Because the first two rivers are part of the Lake Biwa–Yodo River system, the suspected origin of Taiwanese *Z. platypus*, samples from these two rivers are combined in further analyses. The last two rivers are approximately tens to hundreds of kilometers distant from Lake Biwa and belong to distinct river systems (Fig. 1). The sampling scheme provides information regarding the geographic genetic differentiation of Japanese *Z. platypus* and allows us to examine the hypothesis of a Lake Biwa origin of Taiwanese *Z. platypus*. Two fish of *Z. evolans* from northern Taiwan were included as reference samples.

DNA was extracted from muscle tissue following standard phenol-chloroform procedures (Sambrook et al., 1989).

Sequence amplification, sequencing, and data analysis.—A fragment of mitochondrial DNA (mtDNA) including a part of the 5′-end of the D-loop region was amplified using two primers, MtRNA-Thr1F (5′-GTAATCYGAAGATCGGAGGT-3′) and MD-loop1R (5′-AAGAATGCTCGGCATGTGGG-3′) (designed by Ma et al.). Polymerase chain reactions (PCR) (25 μl) were performed containing about 100 ng template DNA, 2.5 μl 10 × *Pfu* reaction buffer, 0.5 μl dNTPs mix (10 mM), 1 μl each primer (10 μM), and 1.25 U *Pfu* DNA polymerase (Promega, Madison, WI, USA). Thermal profiling consisted of 1 min of predenaturation at 94°C, followed by 40 cycles of 94°C denaturation for 1 min, 55°C annealing for 30 s, and 72°C extension for 1 min 30 s, and a final 10 min extension at 72°C. PCR products of each fish were sequenced directly with the PCR primer MtRNA-Thr1 by a DYEamic ET dye terminator kit (Amersham Biosciences, Pittsburgh, PA, USA), following the manufacturer's protocols, and analyzed with a MegaBACE 1000 automated sequencer (Amersham Biosciences).

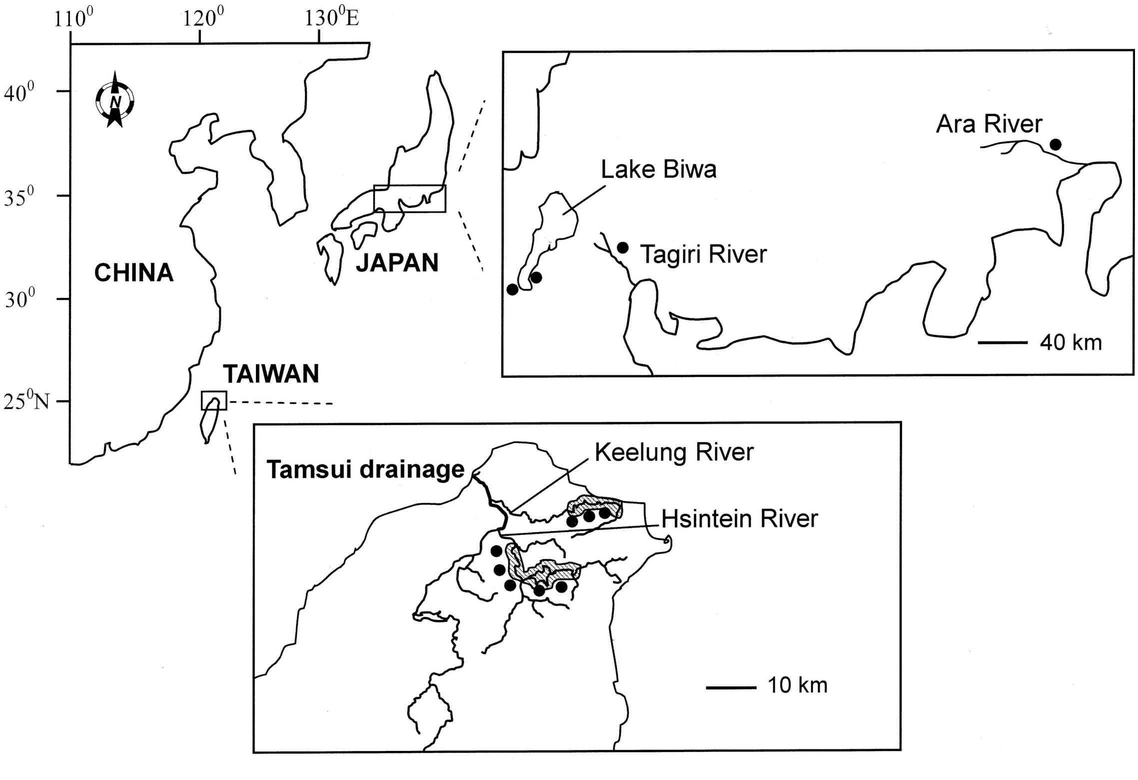

Fig. 1. Map of East Asia and sampling locations (●) of *Zacco platypus* in Taiwan and Japan. *Shaded areas* indicate the distribution of *Z. platypus* in Taiwan

D-loop sequences (495 base pairs, bp) were aligned and corrected by Sequencher 4.0.5 (Gene Codes, Ann Arbor, MI, USA). The number of variable sites and uncorrected *p*-distances were examined using MEGA 3 (Kumar et al., 2004). Haplotype diversity (h) and nucleotide diversity (π) were calculated with Arlequin 2.000 (Schneider et al., 2000). Phylogenetic analysis was performed with PAUP* 4.0b10 (Swofford, 2002) for the neighbor-joining (NJ) method. A sequence evolution model that fits our data for NJ analysis was chosen using a likelihood ratio test as implemented in the program Modeltest 3.06 (Posada and Crandall, 1998). The best-fitting model was estimated to be the Hasegawa et al. (1985) model, with adjustments for invariant sites ($I = 0.9073$) and gamma distribution shape ($G = 0.5739$) (i.e., HKY + I + G model). Bootstrapping with 1000 replications was used to evaluate the reliability of the branching order for the NJ analysis. A statistical parsimony network (Templeton et al., 1992) was constructed using the program TCS version 1.13 (Clement et al., 2000). The limit of maximum mutational steps between haplotypes was set to nine under 95% confidence limits. Gaps in sequences were treated as the fifth state.

Results

Sequence variation and genetic diversity. A total of 22 unique haplotypes were identified among 77 *Zacco platypus* from five populations in Taiwan and Japan. Of 495 base pairs (bp) scored from the mtDNA D-loop region, 21 positions were variable among individuals: 20 sites were transition substitutions, 1 of which also had a single nucleotide deletion, and 1 site was a transversion (Table 1). Estimates of nucleotide divergence (*p*-distance) among haplotypes ranged from 0.20% to 2.82%. For the two *Z. evolans* collected from northern Taiwan, only 1 haplotype was recorded (Table 1). All sequences are available at GenBank (accession numbers DQ377719–DQ377741).

Haplotype distribution of *Z. platypus* revealed a geographic pattern among populations from Japan (Table 1). All haplotypes, except J3, collected from one population were restricted to that population. In contrast, no clear geographic pattern of haplotype distribution was observed in the Taiwanese populations. All haplotypes collected from the Keelung River were also found in the Hsintein River. In particular, four haplotypes (J1–J4) recorded in the Taiwanese populations were shared with samples from Japan: J1–J4 were found in the Lake Biwa population, and J3 was also found in the Ara River population (see Table 1). Haplotype diversity (h) was high in the Taiwanese sample with a mean of 0.852, which was comparable to that among populations from Japan, ranging from 0.718 in the Tagiri River population to 0.909 in the Lake Biwa population (Table 2). Nucleotide diversity (π) among populations ranged from 0.004 in the Tagiri River population to 0.014 in the Ara River population (Table 2).

Phylogenetic analysis. The neighbor-joining phylogenetic analysis for sampled individuals revealed two groups of *Z. platypus* haplotypes (Fig. 2). The first group consisted of haplotypes from Taiwan and from Lake Biwa, the Tagiri River, and the Ara River in Japan. The second group was composed of four haplotypes sampled from the Ara River. This result, together with the high bootstrap support for respective haplotype groups (73% and 98%), suggested a closer phylogenetic relationship between Taiwanese and some Japanese haplotypes relative to that among Japanese haplotypes. However, because relationships among haplotypes within the two major groups were mostly unresolved, phylogenetic inferences about structure among haplotypes were limited (Fig. 2). The parsimony network provided more insight into the relationships among the haplotypes than did the NJ analysis (Fig. 3). Three clades (A, B, and C) with a significant association between genetic and geographical variation were observed among the 22 *Z. platypus* haplotypes (Fig. 3). Most importantly, clade A con-

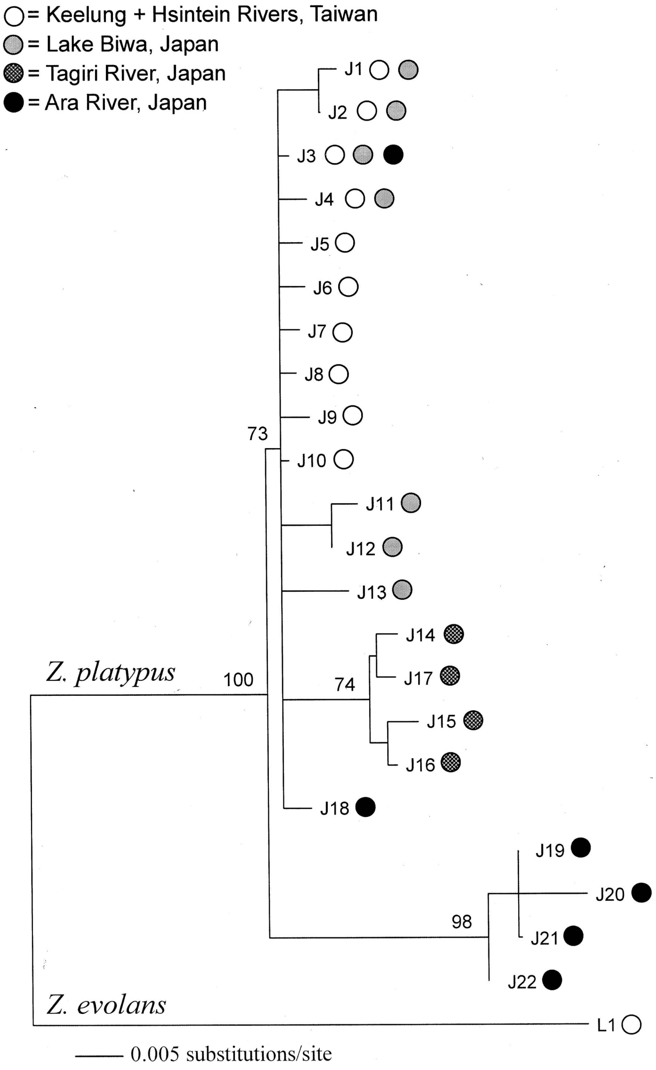

Fig. 2. Neighbor-joining tree (HKY + I + G model) illustrating the relationships of 22 mtDNA D-loop haplotypes of 77 *Zacco platypus* from five populations from Taiwan and Japan and two *Z. evolans* from Taiwan. *Numbers at tree nodes* indicate bootstrap values (only values >70% are shown)

Table 1. Distribution of 23 mtDNA D-loop haplotypes of *Zacco platypus* (Taiwan, $n = 45$ from two populations; Japan, $n = 32$ from three populations) and *Z. evolans* ($n = 2$)

Haplotype	GenBank accession no.	9	28	53	73	78	83	105	107	108	109	156	172	192	193	209	210	211	214	215	235	241	265	268	269	278	283	318	336	351	418	430	431	433	438	442	443	Taiwan		Japan		
																																						Keelung River	Hsintein River	Lake Biwa	Tagiri Rver	Ara River
Z. platypus																																										
J1	DQ377719	A	A	A	G	T	T	A	A	C	C	C	G	A	C	T	C	T	C	C	T	T	A	G	A	G	C	T	T	T	T	G	A	T	C	C	G	4	1	1		
J2	DQ377720	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.		1	2		
J3	DQ377721	.	.	.	.	.	.	–	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.		1	2		3
J4	DQ377722	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	2	11	1		
J5	DQ377723	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	2	3			
J6	DQ377724	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	A	.	A	.	.	.	.	.	.	.	.	.	.	.		2			
J7	DQ377725	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	A	.	A	T	.	.	.	.	.	.	.	.	.	.		5			
J8	DQ377726	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	A	.	.	T	.	.	.	.	.	.	.	.	.	.	1	1			
J9	DQ377727	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	A	.	.	T	.	.	.	.	.	.	C	.	.	.		2			
J10	DQ377728	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	4	5			
J11	DQ377729	.	.	.	A	.	.	–	.	.	.	.	.	.	.	.	T	C	.	.	.	.	.	A	.	A	T	.	.	.	.	.	.	.	.	.	.			3		
J12	DQ377730	.	.	.	A	.	.	–	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	A	.	A	T	.	.	.	.	.	.	.	.	.	.			1		
J13	DQ377731	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	T	C	.	.	.	.	.	.	.	.	.			1		
J14	DQ377732	.	G	.	.	.	.	.	.	T	.	.	A	.	.	.	T	.	.	.	.	C	.	A	.	A	.	.	.	.	.	.	.	.	.	.	.				6	
J15	DQ377733	.	.	G	.	.	.	.	.	T	.	.	A	.	.	.	T	.	.	.	.	C	.	A	.	A	.	.	.	.	.	.	.	C	.	.	.				2	
J16	DQ377734	.	.	.	.	.	.	.	.	T	.	.	A	.	.	.	T	.	.	.	.	C	.	.	.	A	.	.	.	.	.	.	.	C	.	.	.				4	
J17	DQ377735	.	.	.	.	.	.	.	.	T	.	.	A	.	.	.	T	C	.	.	.	C	.	A	.	A	.	.	.	.	.	.	.	.	.	.	.				1	
J18	DQ377736	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	C	.	A	.	A	.	.	.	.	.	.	.	.	.	.	.					1
J19	DQ377737	.	.	.	.	C	.	G	.	.	T	.	A	.	.	.	T	.	G	.	.	.	.	A	.	.	.	.	C	.	.	A	G	C	.	.	.					1
J20	DQ377738	.	.	.	.	C	.	G	.	.	T	T	A	.	.	.	T	.	G	.	.	C	.	A	.	.	.	.	C	.	.	A	G	C	.	.	.					1
J21	DQ377739	.	.	.	.	C	.	G	.	.	T	.	A	.	.	.	T	.	G	.	.	.	.	A	.	.	T	.	C	.	.	A	G	C	.	.	.					1
J22	DQ377740	.	.	.	.	C	.	G	.	.	T	.	A	.	.	.	T	.	G	.	.	.	.	A	.	.	.	.	C	.	.	A	G	.	.	.	.					1
Z. evolans																																										
L1	DQ377741	G	.	.	.	.	C	G	G	.	–	.	A	C	T	C	T	.	.	A	C	.	T	C	C	A	T	.	C	A	G	.	.	.	A	T	A	1	1			

Vertical numbers indicate the variable nucleotide sites within the 495-bp sequence

Hyphens indicate gaps and dots indicate the same nucleotide as in the first haplotype

Numbers under each population indicate the number of sampled individuals with that haplotype

Table 2. Haplotype diversity (h) and nucleotide diversity (π) within populations and mean uncorrected *p*-distances within and between populations, obtained from mtDNA D-loop sequences of *Zacco platypus*

	Haplotype diversity (h)	Nucleotide diversity (π)	Mean *p*-distance (standard deviation)
Intrapopulation			
Taiwan	0.852	0.0048	0.0048 (0.0019)
Japan[a]	0.928	0.0129	0.0121 (0.0018)
Lake Biwa	0.909	0.0082	0.0071 (0.0025)
Tagiri River	0.718	0.0040	0.0040 (0.0018)
Ara River	0.898	0.0140	0.0129 (0.0036)
Interpopulation			
Taiwan vs. Japan[a]	—	—	0.0119 (0.0041)
Taiwan vs. Lake Biwa	—	—	0.0064 (0.0021)
Taiwan vs. Tagiri River	—	—	0.0119 (0.0040)
Taiwan vs. Ara River	—	—	0.0119 (0.0033)
Lake Biwa vs. Tagiri River	—	—	0.0135 (0.0042)
Lake Biwa vs. Ara River	—	—	0.0138 (0.0034)
Tagiri River vs. Ara River	—	—	0.0172 (0.0045)

[a] Pooled samples from Lake Biwa, Tagiri River, and Ara River of Japan

—, not calculated

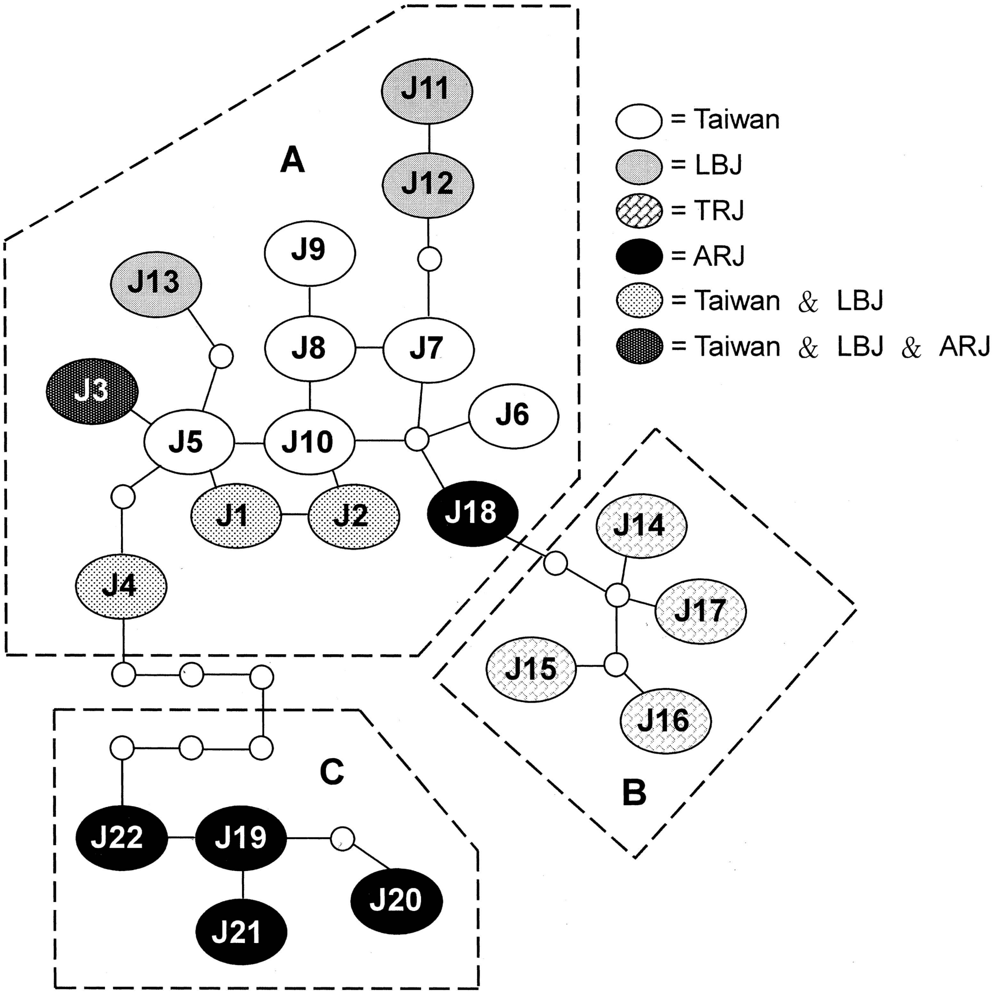

Fig. 3. Statistical parsimony network of 22 *Zacco platypus* haplotypes. *Ovals* represent haplotypes and *circles* represent hypothetical unsampled haplotypes. *Each line connecting two haplotypes* represents a single nucleotide mutation event. Lengths of the connecting lines are not significant. *A*, *B*, and *C* indicate the three major clades with a significant association between genetic and geographic variation among haplotypes. *LBJ*, Lake Biwa of Japan; *TRJ*, Tagiri River of Japan; *ARJ*, Ara River of Japan

sisted of all haplotypes from Taiwan and Lake Biwa. These haplotypes were either closely related to each other or shared by individuals from Taiwan and Lake Biwa. In contrast, clade B and clade C contained haplotypes strictly from the Tagiri and Ara Rivers, respectively, and no Taiwanese samples were related to these haplotypes (Fig. 3).

Genetic divergence among populations. The mean nucleotide differentiation (*p*-distance) between Taiwanese and Japanese populations of *Z. platypus* (0.0119) was as low as that between populations of *Z. platypus* from Japan, ranging from 0.0135 in the Lake Biwa vs. Tagiri River to 0.0172 in the Tagiri River vs. the Ara River (see Table 2). Remarkably, the mean *p*-distance between Taiwanese and Lake Biwa populations (0.0064) was the lowest among all pairwise population comparisons (Table 2).

Discussion

Evidence presented in this study supports the idea of Chen and Chang (2005) that the Taiwanese *Zacco platypus* was introduced from Lake Biwa in Japan. Many Taiwanese individuals shared haplotypes with individuals from Lake Biwa regardless of their prolonged history of geographic isolation. Even the haplotypes unique to Taiwan are closely related to haplotypes sampled in Lake Biwa (see Table 1, Fig. 3). Furthermore, pairwise comparisons also support a closer genetic relationship between Taiwanese and Lake Biwa populations as compared with that among populations from Japan (see Table 2). Of the ten Taiwanese haplotypes, only J3 is shared with individuals from both Lake Biwa and Ara River (Table 1). This result is unexpected because almost all haplotypes collected in one of the Japanese populations are restricted to that population (Table 1). One explanation for this exception is that the J3 haplotype is widespread and, thus, a subsample including such a haplotype would result in the finding of identical haplotypes across multiple populations. Alternatively, the J3 haplotype found in the Ara River might, in fact, originate from Lake Biwa because of artificial introductions of the Lake Biwa fish into the Ara River, as such introductions are common in Japan (Mori and Nagoshi, 1989). We found that two of the six haplotypes (J3 and J18) collected in the Ara River are either shared by individuals of Lake Biwa or closely related to haplotypes of fish from Lake Biwa, which supports this idea (Fig. 3). However, a lack of knowledge concerning the genetic variation throughout the native range of *Z. platypus* in Japan precludes drawing a firm conclusion at this time.

The high haplotype diversity found in Taiwan (Table 1) contradicts conventional wisdom that artificial stocking often decreases genetic diversity as a result of the founder effect (Nei et al., 1975). However, multiple introductions could restore the genetic diversity. For the Japanese populations (i.e., those from Lake Biwa, the Tagiri River, and the Ara River), where a mean of ten fish was sampled from each, all had haplotype diversities greater than 0.7 (Table 2). Therefore, even inadvertent releases of "not-so-large" numbers of *Z. platypus* could explain the high haplotype diversity in Taiwan.

Introduction of exotic species is a major concern for conserving native biodiversity. Conservation units are populations that should not be merged and require separate management to maintain overall biodiversity and the unit integrity. Any arbitrary translocation of these distinct populations might seriously influence their natural evolutionary process and unit survival via genetic introgression or competition. Evidence from a previous report (Chen and Chang, 2005) and our recent field survey has shown a decline of the *Z. evolans* population in the Tamsui drainage, a likely outcome of the recent introduction of *Z. platypus* from Japan. These results reinforce the necessity to curb human introduction of nonindigenous populations. Thus, management priority of *Zacco* in Taiwan should be given to conserving the indigenous units and preventing further expansion of the invasive unit.

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