AFLPs congruent with morphological differentiation of Asian common minnow *Zacco* (Pisces: Cyprinidae) in Taiwan

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Morphological and amplified fragment length polymorphism (AFLP) analyses were used to examine the evolutionary relationships among Asian common minnows (*Zacco* spp.) in Taiwan, where four morphotypes were recently reported. Congruent results from the two sets of analyses indicate that the four morphotypes represent four separate morphometric clades with distinct AFLPs. Consequently, we conclude that four species exist in Taiwan and the systematics of *Zacco* should be revised accordingly. We also discuss conservation implications and offer a key to the four proposed *Zacco* species.

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Introduction

The Asian common minnow *Zacco* is a cyprinid genus of small, colourful fishes that are dominant in the freshwater ecosystems of East Asia, which includes Japan, Korea, China and Taiwan (Bănărescu 1968; Chen 1998). However, since these minnows are morphologically diverse, the species boundaries and taxonomy are still inconclusive and misidentifications are not uncommon (Wang *et al.* 1997). Previous taxonomic studies have reported six *Zacco* species occurring in Taiwan (cf. Shen *et al.* 1993; Chen 1998): *Zacco barbatus* (Regan 1908), *Zacco pachycephalus* (Günther 1868), *Zacco temminckii* (Temminck & Schlegel 1846), *Zacco taiwanesis* Chen 1982, *Zacco evolans* Jordan & Evermann 1902 and *Z. platypus* (Temminck & Schlegel 1846). However, the species originally described as *Z. barbatus* was reclassified into the genus *Candidia* (Jordan & Richardson 1909), and this arrangement has been accepted by most taxonomists. Subsequently, *Z. evolans* was proposed as a synonym of *Z. platypus* (Oshima 1919), and the occurrence of *Z. temminckii* and *Z. taiwanesis* in Taiwan was considered to have resulted from the misidentification of *Z. pachycephalus* (Wang *et al.* 1997). Consequently, of the six nominal species, only *Z. pachycephalus* and *Z. platypus* are currently treated as valid species in Taiwan (Wang *et al.* 1997). *Zacco pachycephalus*, which is endemic to

Taiwan, occurs naturally in the western part of the country, and its range has been recently extended to eastern areas by artificial introductions of unknown origin (Wang *et al.* 1999). A previous allozyme study indicated that the species exhibits a great deal of genetic differentiation among populations, especially between samples from northern/middle and southern Taiwan (Wang *et al.* 1999). *Zacco platypus*, a common East Asian species, is found in Japan, Korea, China and Taiwan, but its range in Taiwan is restricted to the northern region. Genetic heterogeneities of *Z. platypus* among samples from Japan, Korea and Taiwan (Ming 1991) and among samples from different areas of China (Perdices *et al.* 2004; Berrebi *et al.* 2005) have been reported. These results, taken together, imply the possibility that cryptic species exist in both *Z. pachycephalus* and *Z. platypus*.

Recently we discovered distinct morphotypes within currently recognized Taiwanese *Z. pachycephalus* and *Z. platypus*. We also noticed significant morphological differentiation between Chinese and Japanese samples of *Z. platypus*. Knowledge of the taxonomy and phylogenetic relationships of these *Zacco* is, however, incomplete. This is of importance because, at present, common minnows are the major target fish for anglers in Taiwan, at the same time that their population sizes have declined sharply due to environmental perturbations

(cf. Chen 2001). Because of increasing pressure to conserve these species, defining evolutionary units in *Zacco* has become imperative and urgent. Morphological characters, both morphometric or meristic, have been commonly used to distinguish such units in fish (Dynes *et al.* 1999; Rincón 2000; Costa *et al.* 2003). Nevertheless, one interesting question to arise from this approach is whether or not morphological characters can be supported by genetic data that is currently deemed more pertinent in terms of the biological species concept (Mayr 1942). A notable feature of morphological characters is that certain features may not be directly under genetic control but, instead, induced by environmental factors. Such modifications are especially common in fish (e.g. Skúlason *et al.* 1989; Brönmark & Miner 1992; Day *et al.* 1994). Moreover, comparisons based on such traits could erroneously lead to a conclusion of monophyly by clustering paraphyletic or polyphyletic groups. One way to overcome this difficulty is to simultaneously apply morphological and molecular genetic markers to the organisms of interest. Amplified fragment length polymorphisms (AFLPs; Vos *et al.* 1995) are good molecular markers for distinguishing species in these circumstances, not only because the technique is easy to use but also because it permits analysis on a suite of loci presumably spanning the whole genome without requiring prior sequence information (Ellis *et al.* 1997; Mueller & Wolfenbarger 1999).

We performed a combined morphological and AFLP genetic investigation of the genus *Zacco* in Taiwan to estimate species divergence and to study the relationships among morphotypes. First, we describe patterns of differentiation in morphology, including meristic and morphometric characters, and investigate the genetic variation among morphologically distinct entities using AFLP. Second, the possible relevance of the underlying genetic and ecological factors that may be responsible for the morphological variability are discussed. Finally, we compare morphological and genetic differentiation and provide taxonomic resolution of Taiwanese *Zacco*, and we present a key to the proposed evolutionary units.

Materials and methods
Sampling
A total of 175 *Zacco* (5.5–18.1 cm standard length) was collected from 15 major rivers in Taiwan (rivers 1–13, *n* = 165), Japan (river 14, *n* = 7) and China (river 15, *n* = 3) (Fig. 1 and Table 1). Sampling locations were selected to optimize spatial coverage within the distributional range of *Zacco* in Taiwan, including its native range (rivers 1–11) and artificially stocked areas (rivers 12 and 13). The sample included 93 *Z. pachycephalus* and 82 *Z. platypus*. These two currently recognized species have been reported as comprising more than one taxon each (Ming 1991; Wang *et al.* 1999; Perdices *et al.* 2004; Berrebi *et al.* 2005).
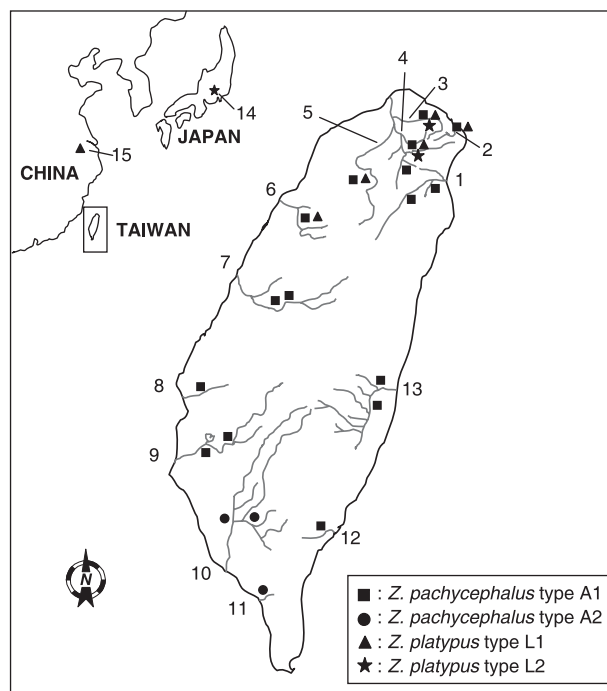
Fig. 1 Map showing sampling locations of the four *Zacco* morphotypes (i.e. type A1, A2, L1 and L2). —1. Lanyang River; —2. Shuangshi River; —3. Keelung River; —4. Hsintien River; —5. Dahan River; —6. Houlung River; —7. Dadu River; —8. Putze River; —9. Tzenwen River; —10. Kaoping River; —11. Fangshan River; —12. Chipen River; —13. Hsiukuluan River; —14. Ara River (Japan); —15. Xinan River (China).

Morphology
Initially, individuals of *Z. pachycephalus* were divided into two morphotypes based on their geographic origins and head sizes: fish from northern, central and eastern Taiwan with a larger head/standard length (HL/SL) ratio of 0.22–0.32 (type A1), and fish restricted to southern Taiwan with a smaller HL/SL ratio of 0.20–0.25 (type A2) (Fig. 2). In contrast, individuals of *Z. platypus* were divided into two morphotypes according to their nuptial colour patterns: blue discrete cross stripes (type L1) and bluish green irregular cross stripes (type L2) (Fig. 2). A peculiar breeding tubercle arrangement was also noted in type L2 males, in which 4–5 large breeding tubercles fused to form a ridge on male cheeks. This is in contrast to individual, round breeding tubercles found on type L1 male cheeks (Fig. 2).

Data on pigmentation, tubercle arrangement, eight meristic (Table 2) and 12 morphometric characters (Fig. 3) were recorded. Among meristic characters, pharyngeal teeth were dissected out and numbers of tooth rows were counted under a dissecting microscope. Numbers of fin rays and vertebrae were counted on double-stained specimens (Wassersug 1976) or X-ray images. For morphometric characters, measurements

Table 1 Details on sample size of *Zacco* used for meristic, morphometric and AFLP analyses, by location and morphotype. Code numbers for sampling locations as indicated in Fig. 1.

Morphotype	Location	Sample size meristic counts	Morphometry	AFLP
Zacco pachycephalus type Al		48	66	39
	1. Lanyang R.	3	6	6
	2. Shuangshi R.	2	4	1
	3. Keelung R.	3	6	1
	4. Hsintien R.	10	10	9
	5. Dahan R.	6	7	4
	6. Houlung R.	5	7	7
	7. Dadu R.	5	5	2
	8. Putze R.	5	5	1
	9. Tzenwen R.	4	8	4
	12. Chipen R.	3	5	3
	13. Hsiukuluan R.	2	3	1
Zacco pachycephalus type A2		17	27	24
	10. Kaoping R.	9	16	13
	11. Fangshan R.	8	11	11
Zacco platypus type L1		24	33	30
	2. Shuangshi R.	4	6	6
	3. Keelung R.	2	2	2
	4. Hsintien R.	9	13	13
	5. Dahan R.	4	6	6
	6. Houlung R.	3	3	0
	15. Xinan R.	2	3	3
Zacco platypus type L2		31	49	30
	3. Keelung R.	8	17	6
	4. Hsintien R.	19	25	17
	14. Ara R.	4	7	7
	Total	120	175	123

were taken from an image of each fish using a digitizer [Summagraphics, MMIII 1201 (Code Micro, CA, USA)], according to Garabana & Saborido-Ray's procedure (http://www.redfish.de/project/deliv02.pdf).

Differences between morphotypes for meristic characters were analysed using univariate statistics. As all meristic characters were independent of standard length (regressions were nonsignificant at $P < 0.01$), no size adjustment of the data was performed. The nonparametric Kruskal–Wallis test was used to identify different meristic characters among morphotypes. Multiple comparisons were carried out using the Dunn procedure (Rosner 2000). All morphometric characters showed a linear relationship with standard length when analysed by specimen (all regressions were significant at $P < 0.01$). Because growth allometry in morphometric characters results in a size effect when comparing fish of different sizes (Reist 1985; Rohlf & Bookstein 1987), morphometric data were statistically adjusted to eliminate size-related variation, thus, permitting comparative analysis in terms of shape. First, morphometric measurements were transformed to common logarithms (i.e. base = 10) in order to homogenize variance. It has been shown that the linearity and normality of morphometric data are usually more closely approached by log-transformed values than by original variables (Hair *et al*. 1998). Then, the common within-type regression coefficients between each log-transformed morphometric character and log-transformed standard length were estimated using analysis of covariance (ANCOVA). Regression coefficients were
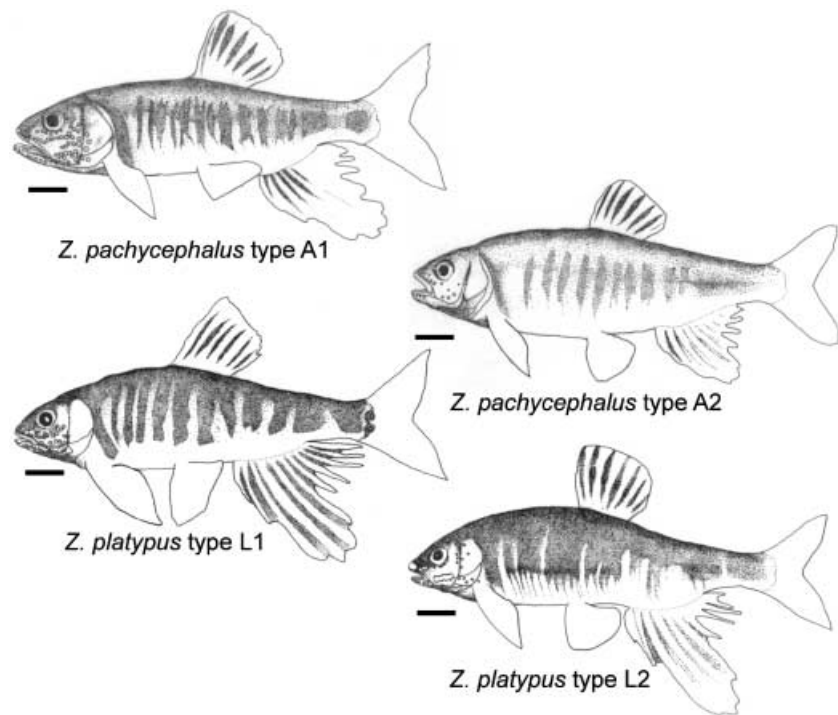

Fig. 2 Lateral view of the four *Zacco* morphotypes from Taiwan: A1 and A2 belong to *Z. pachycephalus*; L1 and L2 belong to *Z. platypus*. Adult males are shown. Scale bars, 10 mm.

Z. pachycephalus type A1

Z. pachycephalus type A2

Z. platypus type L1

Z. platypus type L2

Table 2 Numerical counts and Kruskal–Wallis test for meristic characters of *Zacco* morphotypes A1, A2, L1 and L2. Values are modes (and ranges).

Character	*Z. pachycephalus*		*Z. platypus*		Kruskal–Wallis test		
	Type A1 (*n* = 48)	Type A2 (*n* = 17)	Type L1 (*n* = 24)	Type L2 (*n* = 31)	H	P	Dunn test[a]
Dorsal fin rays*	iii, 7	iii, 7	iii, 7	iii, 7	0.00	1.000	—
Pectoral fin rays*	i, 14 (i, 13–15)	i, 14 (i, 13–14)	i, 14 (i, 13–15)	i, 14 (i, 13–15)	3.19	0.363	—
Pelvic fin rays*	i, 8 (i, 7–8)	i, 8 (i, 7–8)	i, 8 (i, 7–8)	i, 8 (i, 7–8	0.42	0.936	—
Anal fin rays*	iii, 9 (iii, 8–9)	iii, 9 (iii, 8–9)	iii, 9 (iii, 8–9)	iii, 9 (iii, 8–10)	2.49	0.477	—
Predorsal fin scales	22 (20–25)	19 (17–19)	15 (14–18)	17 (16–19)	103.88	< 0.000	A1 A2 L2 L1
Lateral line scales	50 (49–56)	42 (38–48)	44 (42–47)	41 (40–48)	90.09	< 0.000	A1 A2 L1 L2
Vertebrae	40 (40–42)	39 (39–40)	41 (40–41)	42 (40–42)	89.94	< 0.000	A1 L1 A2 L2
Pharyngeal tooth rows	3 (2–3)	3 (2–3)	3	3	8.31	0.040	—

*Roman and Arabic alphabets indicate the numbers of unbranched and branched fin rays, respectively.

[a]Underlined morphotypes indicate no significant difference (*P* > 0.01).
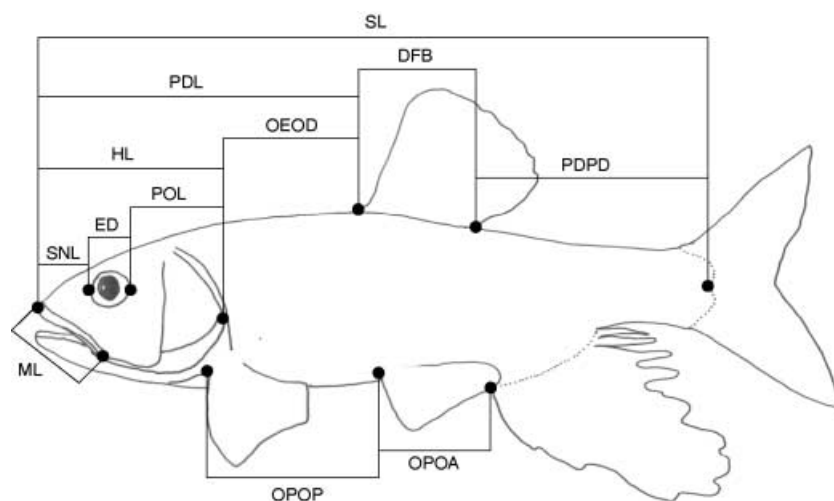

Fig. 3 Morphological characters used for morphometric analysis of *Zacco*. SL, standard length; PDL, predorsal fin length; DFB, dorsal fin base; HL, head length; OEOD, opercular edge to origin of dorsal fin; PDPD, posterior end of dorsal fin to end of caudal peduncle; SNL, snout length; ED, eye diameter; POL, postorbital length; ML, maxillary length; OPOP, origin of pectoral fin to origin of pelvic fin; OPOA, origin of pelvic fin to origin of anal fin.

used for measurement adjustments according to an allometric formula: $AC_i = \log OC_i - \alpha(\log SL_i - \log SL_M)$ (Reist 1985; Rincón 2000; Costa *et al.* 2003), where AC_i is the adjusted logarithmic character measurement of the *i*th fish; OC_i is the original character measurement of the *i*th fish; α is the common within-type regression coefficient of the log C–log SL relationship; SL_i is the standard length of the *i*th fish; and SL_M is the grand mean standard length. A multivariate principal component analysis (PCA) for the allometrically adjusted measurements was performed. The first two principal components (PC1 and PC2) were selected and scored. PC1 and PC2 were then regressed against standard length, and size effect was considered to be removed if regressions were not statistically significant. Difference between morphotypes for each allometrically adjusted measurement was also compared using univariate analysis of variance (ANOVA). Morphotype means were then multiply compared by Scheffe's test (Rosner 2000). The mathematical procedures were executed using the

SPSS statistical package (SPSS Inc. 1997). Unless otherwise indicated, statistical significance was determined at *P* < 0.01.

AFLP

Genomic DNA was extracted from muscle tissue following standard phenol-chloroform procedures (Sambrook *et al.* 1989). AFLP analysis was performed according to Vos *et al.* (1995), with minor modifications. DNA digestion with *Mse*I and *Eco*RI was carried out at 37 °C for 3 h. *Mse*I and *Eco*RI adaptors were subsequently ligated to digested DNA fragments with 200 U T4 DNA ligase at 4 °C overnight to generate template DNA for amplification. Polymerase chain reaction (PCR) was performed in two successive reactions. Pre-amplification reactions used *Mse*I + A and *Eco*RI + A primers, each having one selective nucleotide. The pre-amplification products were then used as templates, after 10-fold dilution in sterile water, for fluorescent selective-amplification reactions using FAM-labelled *Mse*I + ACG and fluorescence-
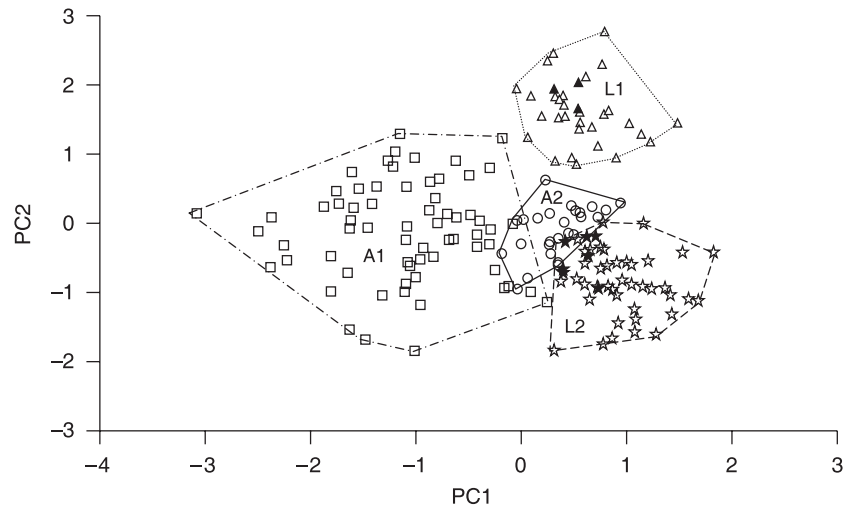
Fig. 4 Plot of individuals on principal components 1 and 2 for *Zacco* types A1, A2, L1 and L2 based on adjusted morphometric characters. Fish collected outside Taiwan are marked solid '▲' and '★' for Chinese and Japanese samples, respectively.

unlabelled *Eco*RI + ACT primers, each containing three selective nucleotides. Amplified DNA was analysed using a MegaBACE 1000 automated sequencer (Amersham Biosciences, PA, USA). A total of 194 AFLP markers, ranging from 46 to 239 base pairs, were scored by using Fragment Profiler 2.0 (Amersham Biosciences). Each AFLP marker was treated as present (1) or absent (0) to create a binary matrix. The matrix was used to estimate the pairwise genetic distance according to Nei & Li (1979) $\{1 - [2a/(2a + b + c)]\}$ and Jaccard (1908) $\{1 - [a/(a + b + c)]\}$, where a is the number of fragments shared by two individuals, and b and c are the numbers of fragments present in either of two individuals, respectively. The resulting distance matrices were used for neighbor-joining (NJ) analyses (Saitou & Nei 1987). Genetic distance matrices were estimated using PhylTools (Buntjer 2000) and the NJ trees were drawn by MEGA 3 (Kumar *et al.* 2004). The reliability and robustness of the tree branch supports were tested by 500 bootstrap replications performed by PhylTools. Two specimens of *Acrossocheilus paradoxus*, which belonged to a different cyprinid subfamily (Barbinae) from the *Zacco* (subfamily: Rasborinae) were used as an outgroup. Calculations of genetic distances among *Zacco* groups, uncovered in NJ analyses, were then carried out with MEGA 3.

Results
Morphology
Significant differences (Kruskal–Wallis test, $P < 0.01$) were found in three of the eight meristic characters among the four morphotypes (Table 2), i.e. numbers of predorsal fin scales, lateral line scales and vertebrae. Posterior comparisons among mean values showed that type A1 has significantly higher numbers of predorsal fin scales and lateral line scales than do the other three morphotypes (Dunn test, all $P < 0.01$). Type L2 has significantly higher numbers of vertebrae and

type A2 has significantly lower numbers of vertebrae when all morphotypes are considered (Dunn test, all $P < 0.01$). Conversely, there was no significant difference in numbers of dorsal, pectoral, pelvic and anal fin rays and pharyngeal tooth rows among the four morphotypes (Kruskal–Wallis test, all $P > 0.01$).

Morphometrically, PCA supports distinction among the four *Zacco* morphotypes. Regressions for PC1 and PC2 against standard length were not significant (PC1: $F_{1,173} = 12.2$, $P < 0.000$; PC2: $F_{1,173} = 49.3$, $P < 0.000$), indicating that size effects had been removed from morphometric characters. Scatter plots of the first two principal components showed four separate groups, each representing a morphotype (Fig. 4). PC1 explained 65.30% of the total variation and type A1 was largely separated from the other types along this axis. In general, type A1 has a larger head and mouth than the other morphotypes as indicated by higher loadings for HL, SNL, POL and ML on PC1 (Table 3). However, the broad scatter of factor scores for type A1 in PCA plots (Fig. 4) indicated considerable variation within this morphotype. This is consistent with the empirical measurements for type A1; for example, HL varied from 21.7% to 31.7% of SL and ML varied from 9.0% to 15.1% of SL among individuals.

PC2 explained 10.72% of the overall variation among morphotypes, mainly reflecting differences in eye size (ED) and extended length of the operculum (POL) (Table 3). An interesting contrast in eye size appears between types L1 and L2 which are well separated on PC2, with L1 being 'large-eyed' and L2 being 'small-eyed' within the nominal species *Z. platypus*. Moreover, the results of the PCA suggest greater morphological differentiation between the two *Z. platypus* morphotypes (L1 and L2) than between the two *Z. pachycephalus* morphotypes (A1 and A2) (Fig. 4). ANOVA for morphometric characters further revealed significant differences in all 11

Table 3 Character loadings and proportions of variance explained by first two principal components (PC1, PC2) for allometrically adjusted morphometric characters of *Zacco*. Abbreviations for morphometric characters as indicated in Fig. 3.

Character	PC1	PC2
PDL	−0.0901	−0.0238
DFB	0.0013	0.0956
PDPD	0.0993	−0.0617
HL	−0.3468	−0.0096
OEOD	0.1577	−0.1067
SNL	−0.4612	−0.1210
ED	−0.0917	0.7050
POL	−0.4463	−0.5452
ML	−0.6438	0.3246
OPOP	0.0393	−0.0682
OPOA	0.0230	−0.2368
% of variance	65.30	10.72

characters among the four types (all *P* < 0.000, Table 4). Subsequent comparisons of mean values revealed significant differences between types A1 and A2 in six characters: HL, OEOD, SNL, POL, ML and OPOP (Scheffe's test, *P* < 0.01), and significant differences between types L1 and L2 in seven characters: DFB, PDPD, OEOD, ED, POL, ML and OPOA (Scheffe's test, *P* < 0.01).

AFLP

A total of 194 loci (fragment sizes 46–239 bp) was scored, of which 143 (73.7%) were polymorphic (at the 5% level) in 123 *Zacco* (Table 1). Every fish examined was unique in terms of its AFLP haplotype, indicating a high degree of overall genetic diversity in *Zacco*. Thirty-one unique fragments were present in only one of the four *Zacco* morphotypes: 11 in type A1, three in type A2, eight in type L1 and nine in type L2. Genetic diversity in terms of percentage polymorphic loci (at

the 5% level) was lowest for type A2 (47.4%), middle for type A1 (54.1%) and type L1 (55.2%), and highest for type L2 (60.3%).

The NJ analysis based on Nei & Li's (1979) distance generated a tree with three main *Zacco* clades (Fig. 5). Clade 1 included all fish of *Z. pachycephalus* (i.e. type A1 + A2), clade 2 consisted of *Z. platypus* type L1 and clade 3 consisted of *Z. platypus* type L2. Within clade 1, two subclades corresponding to types A1 and A2 were identified (Fig. 5). Both subclades were highly supported with a bootstrap value of 75% for type A1 and 72% for type A2. In clade 2, two subclades with low bootstrap support (all < 50%) were identified. Both subclades included fish from Taiwan but only one subclade included individuals from China (Fig. 5). In clades 3, two poorly supported subclades (all < 50%) were also identified, but the two subclades are not geographically mutually exclusive, i.e. individuals from Taiwan and Japan are assigned to each (Fig. 5). The distance parameters used had limited influence on the composition of clades. The Jaccard's (1908) distance yielded a tree similar to that based on Nei & Li's distance. Both distances were lowest for type A1 vs. A2 (Nei & Li: 0.41, Jaccard 0.58) and highest for type A1 vs. L2 and type L1 vs. L2 (Nei & Li: 0.64, Jaccard 0.78).

Key to the morphotypes of Taiwanese **Zacco**

1a. One orange spot on snout tip, body with bluish green irregular cross stripe (the stripe colour is light in females); 4–5 large breeding tubercles fused as a ridge on male cheeks (no breeding tubercles on female cheeks) .... *Z. platypus* type L2
1b. No spot on snout tip, body with ≥ 10 blue discrete cross stripes (the stripe colour is light or obscure in females); separate, round breeding tubercles on male cheeks (no breeding tubercles on female cheeks) 2
2a. One vertical band of blue pigment on caudal peduncle end, body with moderate large scales; pectoral fin rather

Table 4 ANOVA test for log$_{10}$-transformed and allometrically adjusted morphometric characters of *Zacco* morphotypes A1, A2, L1 and L2. Values are mean (and standard error; SE). Abbreviations for morphometric characters as indicated in Fig. 3.

Character	Z. pachycephalus		Z. platypus		ANOVA		Scheffe's test[a]
	Type A1 (*n* = 66)	Type A2 (*n* = 27)	Type L1 (*n* = 33)	Type L2 (*n* = 49)	$F_{(3, 171)}$	P	
PDL	1.654 (0.0013)	1.651 (0.0021)	1.630 (0.0019)	1.630 (0.0015)	71.5	< 0.000	A1 A2 L1 L2
DFB	1.027 (0.0047)	1.044 (0.0073)	1.041 (0.0066)	1.011 (0.0054)	5.9	< 0.000	A2 L1 A1 L2
PDPD	1.550 (0.0014)	1.548 (0.0022)	1.567 (0.0020)	1.586 (0.0016)	108.7	< 0.000	A1 A2 L1 L2
HL	1.385 (0.0033)	1.319 (0.0052)	1.306 (0.0047)	1.294 (0.0039)	126.6	< 0.000	A2 L1 L2 A1
OEOD	1.319 (0.0026)	1.374 (0.0041)	1.341 (0.0037)	1.362 (0.0030)	60.3	< 0.000	A2 L2 A1 L1
SNL	0.822 (0.0064)	0.762 (0.0100)	0.727 (0.0091)	0.719 (0.0074)	44.6	< 0.000	A2 L1 L2 A1
ED	0.749 (0.0038)	0.730 (0.0060)	0.799 (0.0054)	0.703 (0.0044)	66.0	< 0.000	A1 A2 L1 L2
POL	1.078 (0.0043)	0.981 (0.0068)	0.925 (0.0061)	0.977 (0.0050)	167.7	< 0.000	A2 L2 A1 L1
ML	1.019 (0.0053)	0.884 (0.0083)	0.895 (0.0075)	0.827 (0.0061)	202.5	< 0.000	A2 L1 A1 L2
OPOP	1.363 (0.0034)	1.387 (0.0054)	1.362 (0.0048)	1.375 (0.0040)	6.2	< 0.000	A2 L2 L1 A1
OPOA	1.232 (0.0038)	1.223 (0.0059)	1.212 (0.0054)	1.243 (0.0044)	7.4	< 0.000	L1 A1 A2 L2

[a]Underlined morphotypes indicate no significant difference (*P* > 0.01).
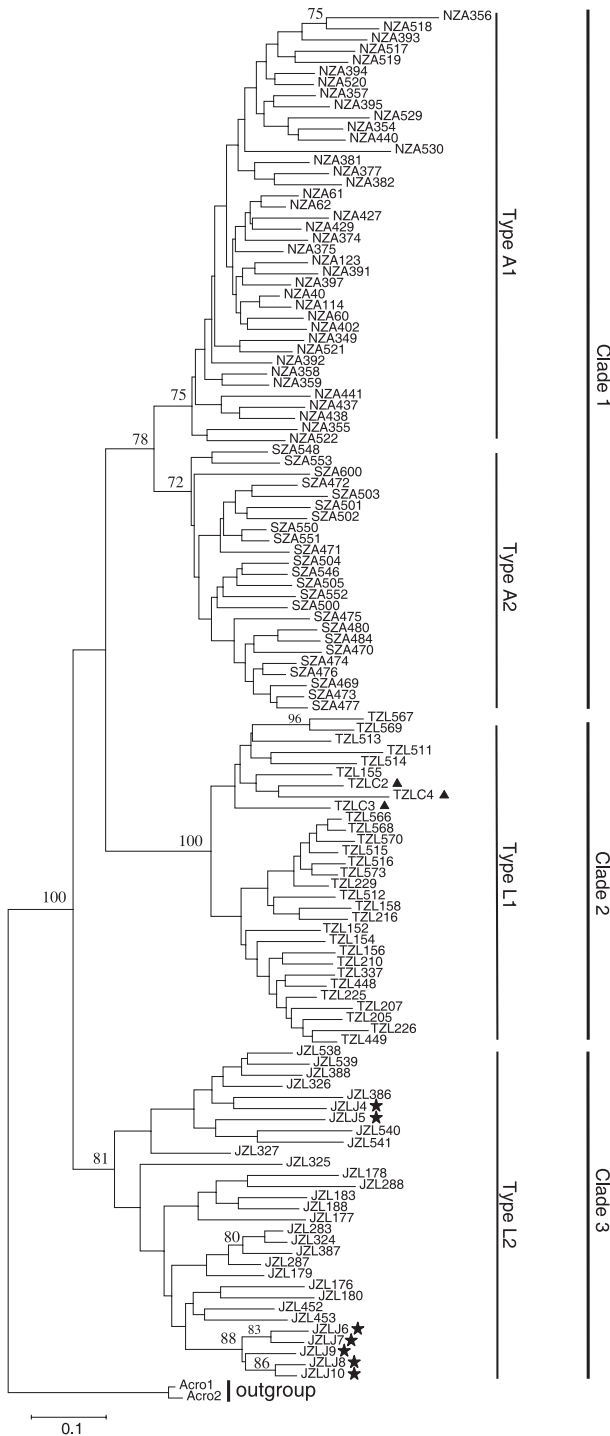
Fig. 5 Neighbor-joining tree of 123 individuals of the *Zacco* morphotypes A1, A2, L1 and L2 based on AFLPs. Genetic relationships were derived from Nei and Li's distance (1979). Numbers at tree nodes indicate bootstrap values > 70%. Fish collected outside Taiwan are marked '▲' and '★' for Chinese and Japanese samples, respectively.

long and tip extending to or beyond the pelvic fin origin in males .... *Z. platypus* type L1

2b. No band of pigment on caudal peduncle end, body with small scales, pectoral fin tip not extending to the pelvic fin origin 3

3a. Twenty to 25 predorsal fin scales, 49–56 lateral line scales, maxillary length generally longer (9.0–15.1% of the standard length), head size generally larger (21.7–31.7% of the standard length); anal fin tip extending to or beyond the caudal peduncle end in breeding males ....*Z. pachycephalus* type A1

3b. Seventeen to 19 predorsal fin scales, 38–48 lateral line scales, maxillary length generally shorter (7.7–9.5% of the standard length), head size generally smaller (19.8–24.7% of the standard length); anal fin tip not extending to the caudal peduncle end in breeding males......................................*Z. pachycephalus* type A2

Discussion

Our results indicate that the four *Zacco* morphotypes (A1, A2, L1 and L2) found in Taiwan are distinct in morphological and genetic characters and should be treated as separate evolutionary units. In contrast, populations within these morphotypes were remarkably similar in terms of their divergence in morphological characters across morphotypes, except for the type A1 which showed considerable variation in some morphological characters.

Genetic basis of morphological variation

Two lines of evidence indicate that genetic differentiation is in accordance with morphological differentiation among the four morphotypes. First, all individuals examined belonged to one morphotypes were clustered into the same AFLP clade with a high bootstrap support. Second, similar forms of morphological variation were found at multiple locations, even among sympatric individuals in northern Taiwan (types A1, L1 and L2) and those of south-western Taiwan (types A1 and A2). If morphotype-specific morphologies are largely the result of environmental factors, similar environmental conditions between relatively close areas would effectively reduce the magnitude of variation. As stated above, both distinctions in morphology and congruence of morphological and genetic data suggest that barriers to gene flow act to maintain the observed morphologies. The gene flow could be reduced by intrinsic mechanisms, such as hybrid incompatibility or hybrid breakdown, or external factors, such as restricted dispersal (see below).

The notable morphological variation in type A1 fish has frequently resulted in their misidentification (cf. Wang *et al.* 1997), although the AFLP data show similar patterns of genomic profiling among individuals (Fig. 5). One explanation of the relatively higher morphological diversity among individuals of this morphotype is that A1 contains higher

genetic variability that is reflected in its morphology. Alternatively, environmental conditions resulting in phenotypic plasticity or the interactions between genetic and environmental factors could cause the high degree of morphological variation observed, as reported in many other fish species (Skúlason et al. 1989; Brönmark & Miner 1992; Day et al. 1994). A high degree of morphological flexibility, in turn, has the advantage of permitting rapid adaptation in different environmental conditions. This may account for the wide distribution of type A1 fish in various freshwater ecosystems (e.g. streams, rivers, pools, reservoirs).

Evolutionary aspect of morphological divergences
The NJ analysis based on AFLP data among the four morphotypes confirmed the close relationship between the two *Z. pachycephalus* morphotypes, A1 and A2 (Fig. 5). This relationship is strongly supported by high bootstrap replications and is concordant with previous allozyme results (Wang et al. 1999). The close relationship and allopatric distribution pattern of these two morphotypes (Fig. 1) suggest that they are vicariant lineages isolated by historical biogeographic barriers near south-western Taiwan. Wang et al. (1999), based on allozymes and geological evidence, proposed that an extremely steep sea trench near the Kaoping estuary may act as a significant historical barrier to speciation (c. 10 000– 6000 years ago), by which *Z. pachycephalus* as well as other freshwater species of fish in Taiwan are currently divided into northern/middle and southern groups. According to this hypothesis, the differentiation (both morphological and genetic) between types A1 and A2 is likely the result of prolonged isolation during this period. We found significant differentiation in head morphology (HL, SNL and POL) and maxillary length (ML) between types A1 and A2 in line with their geographical separation. Type A1 from northern and middle Taiwan is, on average, larger and more robust in these morphological characters than type A2 from southern Taiwan. Head morphology is of considerable importance for feeding and differentiation in the shape of the maxillary often reflects changes in forging mode or diet composition (Skúlason et al. 1989; Langerhans et al. 2003). In the wild, *Zacco* feed on algae, aquatic insects and other invertebrates. Differences in climatic patterns and hydrological conditions between northern and southern Taiwan (Lin & Chou 1974) could influence the community structures of aquatic biota and, thereby, cause varying selection pressures in the functional morphology of fish feeding. Consequently, the morphological differences between types A1 and A2 probably reflect their genetic differentiation as a consequence of prolonged isolation combined with differences in the natural environment and diet.

In contrast to the results obtained for types A1 and A2 of *Z. pachycephalus*, the NJ tree did not support a close relationship between the two *Z. platypus* morphotypes, L1 and L2

(Fig. 5). Type L1 did not group with type L2 but grouped instead with the clade of *Z. pachycephalus* (type A1 + type A2). Furthermore, pairwise comparisons indicated a relatively high genetic differentiation between types L1 and L2, with a Nei & Li's distance of 0.64 and a Jaccord's distance of 0.78. These two morphotypes were separated on the basis of prominent differences in their nuptial colour patterns and breeding tubercle arrangements (Fig. 2). Morphometric analysis further indicated that type L1 is 'large-eyed' and type L2 is 'small-eyed' within the nominal species *Z. platypus* (Fig. 4 and Table 4). Because types L1 and L2 are sympatric in northern Taiwan (Fig. 1), the magnitude of AFLP variation and morphological variation suggest that these two morphotypes are distinct species. Individuals of intermediate morphology were never observed, implying hybridization between these two morphotypes is extremely rare, if it exists at all, in the wild. Nuptial coloration and breeding tubercles are reproductive characters for many fish and it has been suggested that patterns of these characters are important in species recognition and mate choice (Winfield & Nelson 1991; Skarstein & Folstad 1996; Kortet et al. 2004). In both types L1 and L2, the degree of nuptial coloration and extent of tubercle ornamentation are particularly conspicuous in sexually active males, although coloration and tubercle are often unapparent in females and subadults. This implies that nuptial colour patterns and breeding tubercle arrangements may play roles in *Zacco* morphotype recognition and possibly act as reproductive barriers between morphotypes. Even though *in situ* disruptive sexual selection in nuptial colour patterns has been proposed as a common mode of speciation among cichlids (Knight et al. 1998; Seehausen et al. 1998), it seems highly improbable that the divergence of types L1 and L2 was initiated in such a way in northern Taiwan because these two morphotypes are not monophyletic in origin, and are not sister taxa to each other (Fig. 5). Supposing that disruptive selection does facilitate speciation between types L1 and L2 by acting as the initial step in divergence, it would then be expected that these two morphotypes are genetically closely related, and therefore recently diverged. Our Chinese samples of the nominal *Z. platypus* belong to type L1 whereas Japanese samples of nominal *Z. platypus* are type L2. Therefore, the sympatry of type L1 and type L2 in northern Taiwan is of particular interest because outside Taiwan type L1 and type L2 are allopatric (type L1 occurs in China and type L2 occurs in Japan and Korea). Such distributions suggest that the sympatry of type L1 and type L2 in northern Taiwan might result from secondary contact. Therefore, the morphological differences between L1 and L2 could be a consequence of the long independent histories of these morphotypes. We did not detect any major habitat differences between types L1 and L2 in northern Taiwan so environment-mediated divergences might be minor.

Taxonomic and conservation implications

The finding of four genetically distinct morphotypes (A1, A2, L1 and L2) in Taiwanese *Zacco* contrasts with Wang *et al.*'s (1997) taxonomic conclusion that there are only two valid *Zacco* species in Taiwan, *Z. pachycephalus* and *Z. platypus*. Furthermore, types L1 and type L2, which both currently belong to the nominal species *Z. platypus*, do not form a monophyletic group in the AFLP analysis. These results, and the fact that we did not find any hybrid individuals between types L1 and L2 in northern Taiwan, suggest that the current systematic scheme of Taiwanese *Zacco* needs revision.

Zacco pachycephalus is an endemic species of Taiwan. Günther (1868) described *Z. pachycephalus* according to fish from northern Taiwan (Taipei) and gave the characters: (1) 'The length of the head is contained from thrice and a half to thrice and three-fourths in the total length (without caudal)' and (2) 'mouth rather wide, the maxillary extending somewhat beyond the vertical from the front margin of the eye.' Applying these two diagnostic characters and the sampling locality, we find type A1 in agreement with the original description of *Z. pachycephalus*. Therefore, type A1 is recognized as *Z. pachycephalus* (Günther 1868).

Type A2 is not only morphologically distinct but also geographically separated from type A1. Furthermore, the reciprocally monophyletic relationship of the types A1 and A2 should merit a new species status for the type A2, other than the previously reported *Z. temminckii* (Oshima 1919) or *Z. taiwanesis* Chen 1982. Type A2 with ≥ 10 blue discrete cross stripes on both sides of its body does not fit the description of *Z. temminckii*, which has a dark longitudinal band along the body. Although type A2 is similar to *Z. taiwanesis* in having such blue discrete cross stripes, it does not correspond to the reported distributional range of *Z. taiwanesis* ('Choshi River' in middle Taiwan, the range of type A1). Consequently, the type A2 might represent a new taxon which has not yet been described.

Zacco platypus had been long considered a single species across the whole of East Asia (Bănărescu 1968; Shen *et al.* 1993). Our results indicate that two *Z. platypus* morphotypes (types L1 and L2) are sympatric in northern Taiwan and should be treated as different species. Temminck & Schlegel (1846) first described *Z. platypus* based on samples from Japan. The morphological characters designated for this species include a peculiarly striped colour pattern: body with a series of greenish, irregular cross bands (Temminck & Schlegel 1846). Such a striped pattern is typical for 'Japanese *Z. platypus*' (see Nakabo 1993) and for type L2 rather than type L1. Furthermore, in our samples, all fish from Japan belong to type L2 morphologically and genetically. Consequently, we conclude that type L2 is *Z. platypus* as described by Temminck & Schlegel (1846). Jordan & Evermann (1902),

based on morphological differentiation between Taiwanese and Japanese '*Z. platypus*', named a new species *Z. evolans* for Taiwanese samples, but their nomenclature was not followed by other authors. Overall, *Z. evolans* is distinct from *Z. platypus* by having (1) a body with about 12 discrete cross bars and (2) a long pectoral fin the tip of which can reach the middle of the ventral fin (Temminck & Schlegel 1846; Jordan & Evermann 1902). This description is in line with the differences observed between types L1 and L2. The morphometric and meristic measurements of *Z. evolans* (Jordan & Evermann 1902) are within the range of the intratype L1 variation. Furthermore, a figure of *Z. evolans* provided by Jordan & Evermann (1902) resembles type L1, especially in having large eye size and in the stripe pattern. Therefore, we propose that *Z. evolans* may represent type L1. However, further studies, including more extensive collections from throughout *Zacco*'s range as well as the type specimen examinations are needed because type L1 is also distributed in China where several nominal species for the so-called '*Z. platypus*' have been reported (cf. Chen 1998).

In conclusion, the current cyprinid genus *Zacco* is a group exhibiting a great deal of morphological variation, which is reflected in its uncertain and often contradictory taxonomic treatments (cf. Oshima 1919; Bănărescu 1968; Shen *et al.* 1993; Ashiwa & Hosoya 1998; Chen 1998). To address this issue, we have incorporated genetic information into morphological analyses to resolve the relationships among its congeners. The discontinuities observed in both morphological and genetic characteristics signify patterns and processes of speciation in *Zacco*. This is of importance for the taxonomy as well as the conservation of Taiwanese *Zacco* in order to ensure that the evolutionary lineages are recognized and their evolutionary potential conserved. Undoubtedly, the four morphotypes harbouring distinct morphological and genetic characters are separate evolutionary units and should be the focus of conservation efforts. Populations of these units should not be merged and they may require separate management strategies to maintain overall biodiversity. We also agree with Crandall *et al.* (2000) that consideration of ecology is equally important for species conservation, even when morphological and genetic data are available. Thus, comparative studies of life history and behavioural characteristics of these evolutionary units may reveal additional differences among taxa and provide additional information that will prove critical in developing management plans.

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