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Phylogeography of the Endemic Goby, *Rhinogobius maculafasciatus* (Pisces: Gobiidae), in Taiwan

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Hui-Ling Cheng, Shong Huang, and Sin-Che Lee (2005) Phylogeography of the endemic goby, *Rhinogobius maculafasciatus* (Pisces: Gobiidae), in Taiwan. *Zoological Studies* **44**(3): 329-336. Genetic diversity within and among populations of the spot-banded goby, *Rhinogobius maculafasciatus*, in Taiwan was studied by analyzing mitochondrial DNA sequences. The sequence length varied from 2124 to 2126 bp and included the complete cytochrome b gene, 2 tRNA genes, and the control region of mitochondrial DNA (mtDNA). Forty-one haplotypes were identified from 60 specimens. Sequence analysis indicated that 2 distinct clades exist in *R. maculafasciatus*, and that each clade is divided into 2 subgroups. The 3 populations of the Kaoping River were not included in the same clade. The population sampled from the Lanyang River in northeastern Taiwan probably originated from the eastern coast of China and is connected with populations in southwestern Taiwan by the Coastal Current which flows along the western coast of Taiwan. A hierarchical examination of 6 populations in 3 drainage basins using analysis of molecular variance indicated high genetic differentiation (68.37%) among populations within basins. The results support the hypothesis that the current genetic structure was strongly affected by changes in drainage patterns due to geomorphological processes that occurred in the recent past when the main island of Taiwan formed and separated from the Asian continent 0.55 My before the present.
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Members of the *Rhinogobius brunneus* species complex (Gobiidae), which includes *R. rubromaculatus* and some closely related members of the genus *Rhinogobius*, are widely distributed and common freshwater fishes in Taiwan. According to the time-series changes in strontium (Sr)-to-calcium (Ca) ratios from the primordium to the edge of the otolith, species in the complex are grouped into the 2 life history-based categories of fluvial and amphidromous types (Shen et al. 1998). The fluvial fish was recognized and described as a distinct species, *R. rubromaculatus*, based on morphological and molecular (allozyme) evidence by Lee and Chang (1996). Furthermore, recent detailed examinations have revealed that at least 8 species included in the complex should be considered to occur in Taiwan based on morphometric data and some ecological considerations (Chen and Shao 1996). Nevertheless, molecular evidence of the *Rhinogobius* is scant or lacking.

Rhinogobius maculafasciatus is an endemic freshwater fish found only in middle and lower drainages of southern Taiwan, such as the Tsengwen and Kaoping Rivers (Chen and Shao 1996). This species can be distinguished by the lower counts of longitudinal scale rows and scales between the original 1st dorsal fin and pectoral fin among all species of the *R. brunneus* complex from Taiwan. However, the population genetic structure has not been described to date. During

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the course of field trips for extensive collection of freshwater gobies throughout the island, we found a new population in the Lanyang River of northeastern Taiwan.

The evolutionary origin of the population in the Lanyang River is unclear. A discontinuous distribution makes *R. maculafasciatus* a good candidate for investigating the dispersal capability of this fish in the region. According to the distribution of freshwater fishes, the island of Taiwan can be divided into 3 zoographical districts: (i) the eastern district, (ii) the southern district, and (iii) the northcentral district (Tzeng 1986). Some authors have suggested that the occurrence of most freshwater fish species of marine origin in northern Taiwan was probably due to the strong Kuroshio Current which flows from the south, with only a few of those coming from the east coast of mainland China (Chen and Fang 1999). Some studies have stated that marine and estuarine species often show patterns of species distribution and genetic variation in western Taiwan similar to those in northern Taiwan due to the Coastal Current which flows along the western coast of Taiwan (Chang et al. 2002, Yu et al. 1999, 2002).

A species with a dispersal capacity that connects spatially separated populations and which occurs on either side of a potential barrier to gene flow would be useful when examining intrinsic and extrinsic factors that determine a population's genetic structure. Mitochondrial DNA (mtDNA) analysis has proven to be an especially powerful tool for investigating underlying biogeographic patterns of many taxa and is particularly well suited for providing informative markers at the intraspecific level (Avise 2000). Under the hierarchical model, it is expected that the greatest genetic similarity will be found between populations within a stream, and the greatest differentiation will be between populations in different river drainages. These expectations therefore provide the null hypothesis for which to test the relative importance of the contributions of contemporary gene flow and historical geomorphological events to the observed genetic structure. It is generally accepted that widespread drainage rearrangement has occurred in southern Taiwan within the past 0.5 My (Lin 1966), and this may have been a dominant factor influencing the genetic structure of populations of fish in streams of this region.

In this study, we used the complete cytochrome b gene, 2 tRNA genes, and the control region of mtDNA as markers to estimate the phylogeographical patterns and genetic structure of *R. maculafasciatus*. This study was undertaken with the goal of characterizing the distribution of molecular genetic variations within and among populations of *R. maculafasciatus*. We investigated the (1) genetic variability among populations and (2) the phylogeographic patterns at the hierarchical scale among both geographical regions and populations. The results indicated that the current genetic structure in western Taiwan is more similar to those in northern Taiwan than to those in southern Taiwan and was strongly affected by changes in drainage patterns due to geomorphological processes that occurred in the recent past when the main island of Taiwan formed and separated from the Asian continent.

MATERIALS AND METHODS

Sample collection

In total, 60 individuals of *R. maculafasciatus* were collected from 6 sites within 3 drainage basins in Taiwan (Fig. 1). Details of sampling localities and numbers of specimens for the mtDNA analysis are given in table 1. All specimens were taken by electro-fishing and were preserved in 70% ethanol.

DNA extraction, PCR, and sequencing

Crude DNA was extracted from 0.05 g of skeletal muscle tissues using a GENTRA genomic DNA purification kit (Gentra, Minneapolis, MN, USA). A fragment of approximately 2124 bp of mtDNA, including the complete cytochrome b gene, 2 tRNA genes, and the D-loop region, were amplified. The primers, CY1 (5'-YYTAACCRRGACYAATGACTTGA-3') and D2 (5'-CCGGAGTATGTAGGGCATTCTCAC-3'), were designed from those fishes (*Crossostoma lacustre*, Tzeng et al. 1992; *Cyprinus carpio*, Chang et al. 1994; *Oncorhynchus mykiss*, Zardoya et al. 1995; and *Polypterus ornatipinnis*, Noack et al. 1996). Each 100-μl PCR included 10 ng of template DNA, 20 pmol of each primer (CY1 and D2), 8 μl dNTP (2.5 mM each), 10 μl 10X buffer, and 2.5 U *Taq* polymerase (Takara, Shiga, Japan). The amplification conditions were as follows (35 cycles): denaturation at 94°C for 1 min, annealing at 50°C for 1 min, and elongation at 72°C for 105 s, followed by a final extension at 72°C for 10 min and storage at 4°C. PCR products were purified using Viogene spin columns (Viogene, Taipei, Taiwan). The

amplified DNA was directly sequenced on an automated DNA sequencer (ABI PRISM 377; Applied Biosystems/Perkin Elmer, Boston, MA, USA) using a fluorescence dye terminator cycle sequencing kit (Bigdye, Boston, MA, USA).

Alignment and analysis

Sequences were aligned using published sequences of *R. giurinus* and *R. maculafasciatus* from GenBank with the Lasergene software (DNASTAR, Madison, WI, USA) and the BioEdit program 5.0.9 (Hall 1999). Haplotype diversity (*h*), nucleotide diversity (π), pair-wise genetic distances, and Tajima's D statistic (Tajima 1989a) were computed in the program ARLEQUIN 2.0 (Schneider et al. 2000). Subsequently, this program was also used to calculate the genetic distances between populations and to perform the following computation of analysis of molecular variance (AMOVA), F_{ST} statistics, and mismatch distributions. Samples were grouped and subjected to a hierarchical analysis of variance (AMOVA) (Excoffier et al. 1992) to ascertain whether the group structure observed is significant. F_{ST} values from the pairwise analyses were estimated as measures of population differentiation. The significance of the respective fixation indices (F_{ST}, F_{SC}, and F_{CT}) was tested using permutation procedures described in Excoffier et al. (1992) with 1000 permutations. In addition, F_{ST} values between pairs of populations were calculated, and their significance was also tested using the permutation procedure. Rogers' (1995) parameters of the mismatch distribution or demographic expansion (τ, θ_0, and θ_1) and their respective 95% confidence intervals (CI) were calculated following Schneider and Excoffier (1999). In addition, Harpending's (1994) raggedness index was calculated and mismatch distributions were calculated for pooled pop-

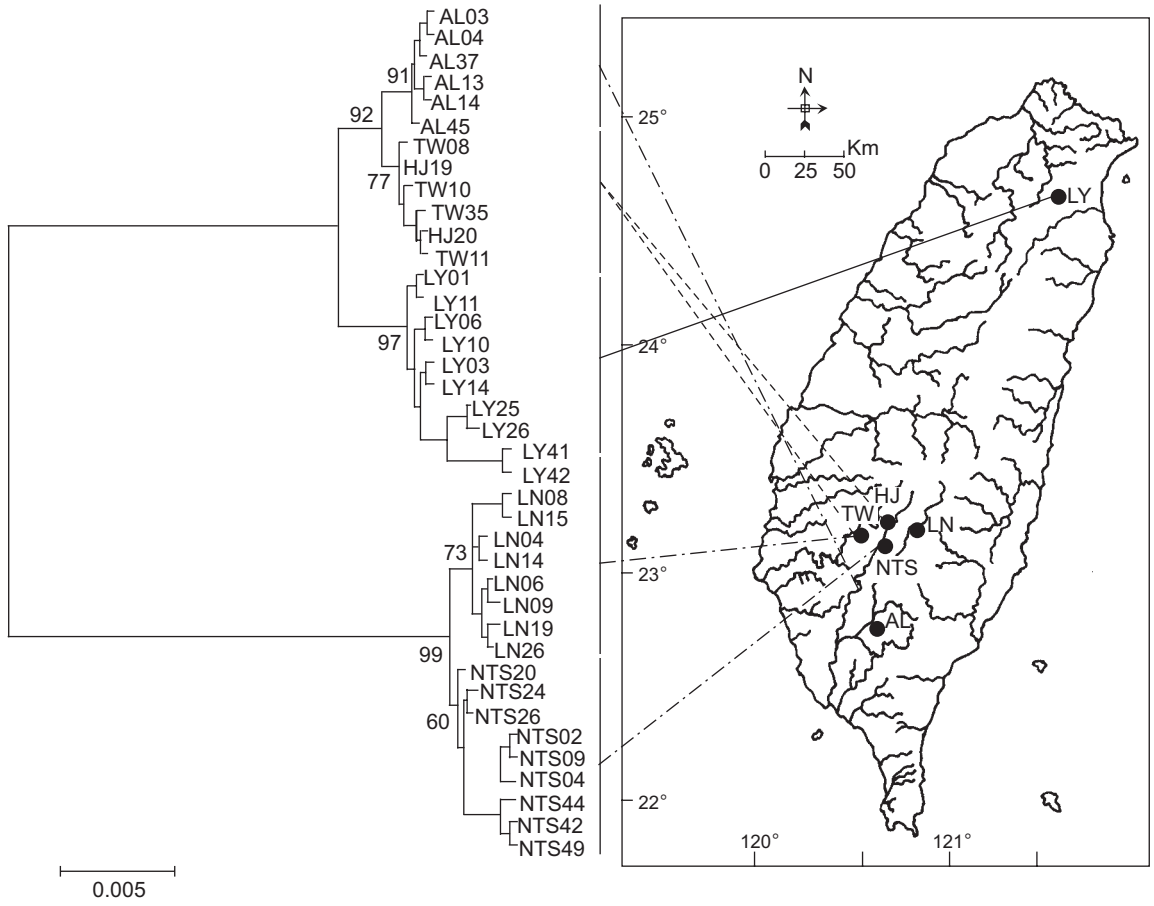

Fig. 1. Map of Taiwan with the stream sampling sites for *Rhinogobius maculafasciatus*. The Neighbor-joining tree was constructed based on comparisons of the sequence alignment of the complete sequence of the cytochrome b gene, tRNA genes, and the control region of mtDNA. Bootstrapping values were estimated from 1000 replicates; only numbers exceeding 50% are shown for each node. AL, Ailiao R.; HJ, Houjyue R.; LY, Lanyang R.; LN, Laonong R.; NTS, Nantsushian R.; TW, Tsengwen R.

ulations from each region and for the total sample.

The parameters that apply to the Neighbor-joining (NJ) analysis were suggested by MODELTEST 3.06 (Posada and Crandall 1998), and that analysis was performed using the Molecular Evolutionary Genetics Analysis program (MEGA, vers. 2.0, Kumar et al. 2001). The reliability of the reconstructed clades was tested by bootstrapping with 1000 replicates. The number of mutations between haplotypes in the pairwise comparisons was also used to construct a minimum spanning network with the aid of the MINSPNET program (Excoffier and Smouse 1994). The hypothesis of random distribution of the haplotypes over geographical areas was tested using permutation analysis of a Chi-square contingency table with 1000 replicates, treating sample locations as categorical variables by Geodis (Posada et al. 2000). Interpretation of the results followed the inference key given in Templeton (1998).

RESULTS

Total complete sequences of 2124~2126 bp in length of mtDNA including 1140 bp of the cytochrome b gene, 71 bp of the threonine tRNA gene, 71 bp of the proline tRNA gene, and 842~844 bp of the control region, resulting in 41 putative mtDNA haplotypes were revealed from 60 individuals of *R. maculafasciatus*. All of those but one which was found in both the Houjyue (HJ) and Tsengwen Rivers (TW) were location specific. Differences betweens haplotypes were due to substitutions: 48 sites were found to be variable (with a transition/transversion ratio of 3.1), and 7 indels were recorded. The genetic variability is shown in table 1. Haplotype diversity was remarkably high within most populations and ranged from 0.6000 (HJ) to 0.9697 (LY). The nucleotide diversity was low and ranged from 0.0006 (AL and HJ) to 0.0044 (LY). At all sites, Tajima's D statistic did not significantly differ from values expected under neutral conditions ($p > 0.05$).

The nucleotide substitution model was examined using MODELTEST 3.06. The parameters for constructing the Tamura-Nei (Gamma) (TrN+G) distances among the 41 haplotypes were obtained and used to construct the NJ dendrogram. The overall sequence distance between local populations of *R. maculafasciatus* was from 0.0009 to 0.0441 (Table 2). No consistent pattern existed between pairwise F_{ST} values and geographical distances. The NJ tree (Fig. 1) reveals 2 divergent clades represented by high bootstrap values of 99. The dendrogram shows that the 3 populations of the Kaoping River drainage, including AL, LN, and NTS, were divided into 3 subclades. Two populations (HJ and TW) in the Tsengwen River drainage formed a highly supported monophyletic group, and were grouped into a clade with the AL population with a bootstrap value of 92. This is a sister clade to the LY population of northern Taiwan.

The hierarchy initially used in the analysis of molecular variance was based on drainage boundaries and consisted of 3 levels: (i) variations within sites; (ii) variations among sites within drainage; and (iii) variation among drainages. Results of the AMOVA are presented in table 3a. The F_{ST} value calculated for all *R. maculafasciatus* was 0.926. Most of the variation was due to differences among populations within basins (68.37%), while 24.22% of the variation was assigned to differences among basins, whereas only 7.41% was

Table 1. Sampling localities by zoographical district, major basin, and population. Localities are followed by a population code, the number of individuals surveyed (*n*), number of unique haplotypes, haplotype diversity (*h*), and nucleotide diversity (π)

Zoographical district	Basin	Population	Code	*n*	Haplotypes	*h*	π
(I) Southern district	Kaoping R.	Ailiao R.	AL	10	6	0.8889 ± 0.0754	0.0006 ± 0.0005
		Laonong R.	LN	12	8	0.9091 ± 0.0649	0.0021 ± 0.0012
		Nantsushian R.	NTS	12	9	0.9545 ± 0.0467	0.0025 ± 0.0015
			Total	34	23	0.9750 ± 0.0125	0.0192 ± 0.0095
	Tsengwen R.	Houjyue R.	HJ	5	2	0.6000 ± 0.1753	0.0006 ± 0.0005
		Tsengwen R.	TW	9	6	0.8333 ± 0.0980	0.0008 ± 0.0006
			Total	14	8	0.8242 ± 0.0703	0.0007 ± 0.0006
(II) North-central district	Lanyang R.	Lanyang R.	LY	12	10	0.9697 ± 0.0443	0.0044 ± 0.0024
All populations				60	41	0.9819 ± 0.0067	0.0227 ± 0.0110

explained by differences within populations. The source of variation among populations within basins was due to the 3 populations of the Kaoping River which were divided into 2 separate clades. Within the Kaoping River basin, 90% of the genetic variance was observed among clades (Table 3b).

Parameters of the mismatch distribution for each population and all populations pooled did not significantly differ from a sudden expansion model. Estimates for the parameters used to estimate the age of expansion (τ) ranged between 1.56 and 6.67. However, owing to the large 95% CI around the mean estimates, there was no statistically significant difference between these estimates.

To rearticulate the phylogeny of the haplotypes of *R. maculafasciatus*, a minimum spanning network reconstructed using mutational changes between mtDNA haplotypes is shown in figure 2. The reconstructed minimum spanning network diagram using MINSPNET largely agreed with the NJ tree of MEGA. It is noticeable that haplotypes of AL were located at the interior nodes of the network that were linked to haplotypes from 2 distinct groups (NTS and LN, and TW and LY). Categorical analyses of associations between clades and geography resulted in rejection of the null hypothesis of no geographical association for 1-step clades (haplotypes) nested in clade 2-1 ($\chi^2 = 50.00$, $p < 0.005$). The inference key of Templeton (1998) was restricted gene flow with isolation by distance.

Table 2. Tamura-Nei (Gamma) genetic distance (below the diagonal) and pairwise F_{ST} estimates (above the diagonal) between populations of *Rhinogobius maculafasciatus* based on mtDNA variations

	AL	LN	NTS	HJ	TW	LY
AL	—	0.9654***	0.9589***	0.8214***	0.7768***	0.7515***
LN	0.0427	—	0.5151***	0.9597***	0.9626***	0.9228***
NTS	0.0429	0.0042	—	0.9513***	0.9558***	0.9169***
HJ	0.0034	0.0425	0.0427	—	0.1585**	0.6636***
TW	0.0036	0.0431	0.0433	0.0009	—	0.6984***
LY	0.0096	0.0434	0.0441	0.0080	0.085	—

Site codes are given in table 1. ** $p < 0.01$; *** $p < 0.005$.

Table 3. Analysis of molecular variance (AMOVA) for mtDNA variations in (a) *Rhinogobius maculafasciatus* and (b) the Kaoping River basin

(a)

Source of variation	d.f.	Sum of squares	Variance components	Percent (%) of total variance	*p* value
Among basins	2	691.548	7.4264	24.22	< 0.001
Among populations within a basin	5	615.081	20.9626	68.37	< 0.001
Within populations	41	122.639	2.2711	7.41	< 0.001
Total		1429.267	30.6601		

(b)

Source of variation	d.f.	Sum of squares	Variance components	Percent (%) of total variance	*p* value
Among clades	1	580.322	39.0068	89.58	< 0.001
Among populations within a clade	2	33.333	2.6180	6.01	< 0.001
Within populations	22	59.433	1.9172	4.40	< 0.001
Total		673.089	43.5420		

An estimate of the average of nucleotide substitutions (K) between haplotypes was 0.0231 ± 0.022. Assuming evolutionary rates of between 2% and 5% changes per 10^6 years for fishes as a reference (Penzo et al. 1998, Dawson et al. 2002), the range of divergence times among populations of *R. maculafasciatus* was 0.49~1.23 My before the present. On average, haplotypes in Taiwan shared a most-recent common ancestor 0.87 Mya.

DISCUSSION

Based on results of the AMOVA, with most of the genetic variation found between streams within drainages rather than among drainages, it is clear in this particular case that there is conformity to the hierarchical model of gene flow. Consequently, it may be inferred that contemporary dispersal of *R. maculafasciatus* has not played a dominant role in structuring populations between the Kaoping and Tsengwen Rivers. Therefore it is useful investigating the processes that have given rise to the observed structure.

Given the high potential for dispersal by this species in terms of its widespread distribution and broad habitat preferences, it is surprising that populations of *R. maculafasciatus* within the Kaoping River display a high degree of differentiation (Fig. 1), which suggests little or no gene flow. Although the level of nucleotide diversity between the 2 clades ranged from 0.0427 to 0.0429 (Table 2), the difference was still within the population level (Chen et al. 1998). Two possible reasons may explain such a phenomenon in *R. maculafasciatus*. First, there are 2 life history-based types, namely landlocked and amphidromous, which are frequently observed in species of *Rhinogobius* (Tzeng and Lin 1996, Shen et al. 1998). Second, it is possible that the 2 groups do not share a common origin. In order to stop the damage caused by flooding, humans altered the streamway of the upper Ailiao River which is connected with the Kaoping River some 70 years ago, but that is hardly sufficient time for gene flow to occur.

According to the distribution of primarily fresh-

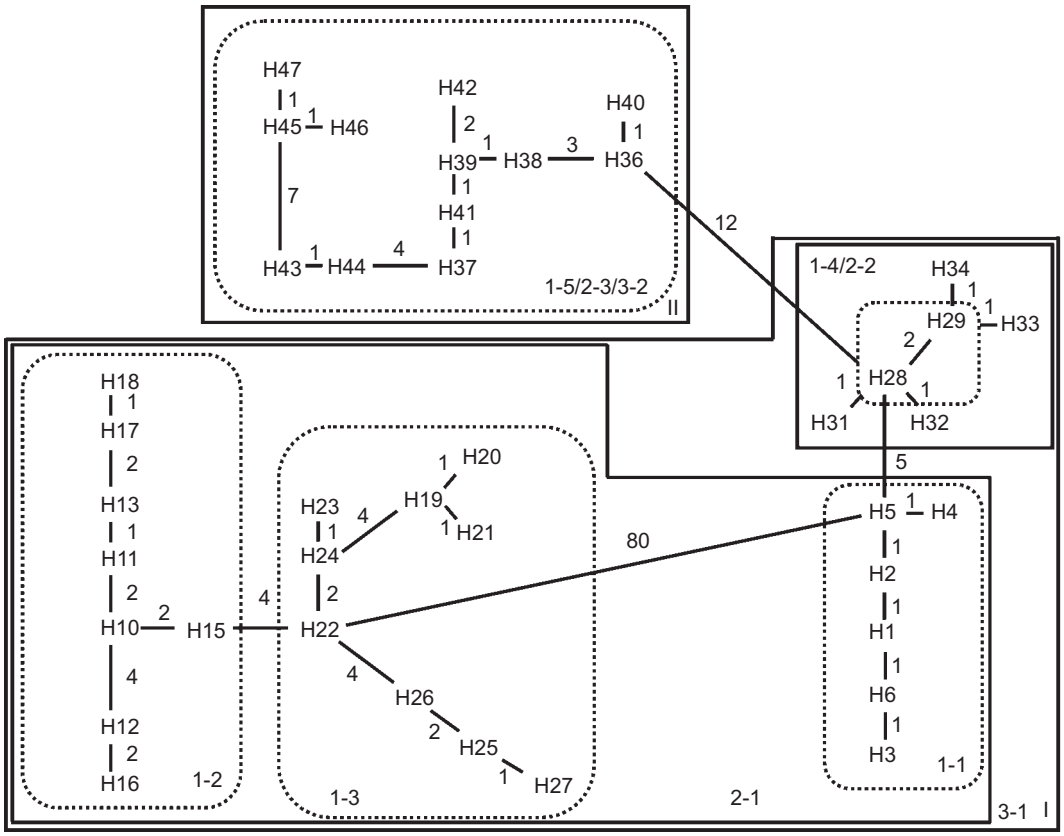

Fig. 2. Minimum spanning network for *Rhinogobius maculafasciatus* haplotypes. Numbers at the nodes indicate the number of nucleotide changes between haplotypes. AL, Ailiao R.; HJ, Houjyue R.; KP, Kaoping R.; LY, Lanyang R.; LN, Laonong R.; NTS, Nantsushian R.; TW, Tsengwen R. I, southern district, and II north-central district.

water fishes, most species are restricted to the western part of Taiwan (Tzeng 1986, Wang et al. 1999 2000). Such a distribution pattern has been thought to be highly correlated with topographical isolation due to the Central Mountain Range which uplifted about 10^6 years ago (Lin 1966). Among freshwater fishes, *R. maculafasciatus* is localized in southern of Taiwan except for the LY population. Our results indicate a sister-group relationship between LY and AL+TW. According to the coalescence theory, the interior position in the minimum spanning network and higher frequencies in populations suggest that the AL group likely represents an ancestral clade that has a greater probability of producing mutational derivatives and may represent some relict ancestral genotypes.

Pairwise *Nm* values deduced from F_{ST} between most populations were smaller than 1, indicating restricted gene flow between populations. In contrast, in spring and summer, the southwestern monsoon prevails and induces a warm coastal current flow northward into the Taiwan Strait from the South China Sea, causing water temperatures to increase from south to north along the western coast; in autumn and winter, there is a cold current flow in the opposite direction, and a branch of the warm Kuroshio Current stops the cold China Coastal Current in the middle of the Taiwan Strait (Chu 1963). *Rhinogobius maculafasciatus* of the AL and TW populations spawns in late spring, and the larvae drift northwards with the coastal current toward the northern part of the Taiwan Strait. In the LN and NTS populations of the upper Kaoping River, *R. maculafasciatus* spawns in autumn, and thus larvae cannot effectually disperse. Furthermore, *R. candidianus* is the dominant species in north-central Taiwan, which of restricts the ability to colonize more-northerly areas by *R. maculafasciatus*. In our sampling, *R. maculafasciatus* and *R. candidianus* are only sympatric in the Tsengwen River drainage, with ratios of 1:3 in TW and 1:8 in HJ.

Analyses of mismatch distributions of *R. maculafasciatus* mtDNA suggested populations at demographic equilibrium. The results of our study revealed a surprising degree of genetic divergence among populations within basins. This study was somewhat limited as to the extent of the current geographical range of *R. maculafasciatus* we examined and was limited by small within-basin sampling in some basins. The discovery of 2 highly divergent haplotype clades in the Kaoping River system was the result of much more extensive sampling within this basin. Equally intense sampling of other drainages is warranted and would surely reveal greater genetic diversity. Certainly, a more-encompassing study across the entire range of *R. maculafasciatus* would reveal greater biodiversity. Further, definitive taxonomic and biogeographic assessments of the *R. brunneus* species complex, a widespread and highly subdivided species, will form an important contribution to understanding the historical biogeography of East Asia.

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