Materials and Methods

Sample collection

A total of 263 *T. viridipunctatus* and *T. luyeanus* were captured during 2004 to 2006. The potential contact zone is around Sinchen, near the Taroko Gorge, and extends 2.5 kilometers to the south and north of the Liwu River (Figure 1). Collected samples were mapped with GPS (GARMIN GPS V). The geographic coordinates of all collected individuals in the adjacent area of the Liwu River are N 24° 08’ to N 24° 10’, E 121° 36’ to E 121° 38’. Samples of *T. viridipunctatus* distributed adjacent to the potential contact zone were collected from Heren (11 individuals), while *T. luyeanus* samples are from Shoufen and Wanrong (25 individuals),

Collected samples were stored in 100% ethanol. DNA was extracted from tail tissues using a modified LiCl method (Gemmell & Akiyama 1996) and stored in a -20◦C freezer.

Mitochondrial DNA typing

Mitochondrial cytochrome *b* sequences were used to determine the maternal haplotype. Two sets of primers were designed by species-specific sequences, to amplify a partial fragment from *T. viridipunctatus* and *T. luyeanus*. The sequences of these two sets of primers are listed: NL: 5’-TTG TAG AGT GAG TAT GGG GG-3’ and NH: 5’-TTG TTT TGA TAA ATG AGT GA-3’, for *T. viridipuctatus* and EL: 5’-TCG TAG AGT GGG TAT GAG GC-3’ and EH: 5’-TTG CTT TGA TAG GTG AAT AT-3’ for *T. luyeanus*. Polymerase chain reaction

(PCR) were conducted in a 10 μL reaction volume containing about 50 ng genomic DNA, 0.2 μM each primer, 0.625 mM dNTP, 1 X PCR buffer (10 mM Tris-HCl, pH 9.0; 50 mM KCl, 0.01% (w/v) gelatin, and 0.1% Triton X-100), 0.4 U *Taq* DNA polymerase (Amersham Biosciences) and 1.5 mM $MgCl_2$. The thermocycler conditions were set to 94 ◦C for 3 min, then 30 cycles of 94 ◦C for 30 s, 50 ◦C for 30 s and 72 ◦C for 30 s, followed by 72 ◦C for 3 min (Lin 2004). Under such condition, each individual was expected to be amplified by only a single set of primers. If any individual was amplified by both sets of primers, the amplified fragment would be sequenced, aligned, and compared to sequences of *T. viridipunctatus* and *T. luyeanus* (Lin 2003).

Microsatellite genotyping

Thirteen microsatellite loci (Lin *et al*., 2006) were applied in detecting hybridization of the nuclear genome. PCR was conducted in a 10 μL reaction volume containing about 50 ng genomic DNA, 0.2 μM of each primer, 0.625 mM dNTP, 1 X PCR buffer (10 mM Tris-HCl, pH 9.0; 50 mM KCl, 0.01% (w/v) gelatin, and 0.1% Triton X-100), 0.4 U *Taq* DNA polymerase (Amersham Biosciences). The thermocycle conditions were set to 94 ◦C for 3 min, then 30 cycles of 94 ◦C for 30 s, 58 ◦C for 30 s and 72 ◦C for 30 s, followed by 72 ◦C for 3 min (Lin *et al.* 2006).

Analysis 1: The PCR products were electrophoresed on a MegaBACE 1000 autosequencer (Amersham Biosciences) using ET-400 (Amersham Biosciences) as size standards. Sizing of alleles was analysed with the software genetic profiler 2.0 (Amersham Biosciences). We calculated the observed and expected heterozygosities using the software

Cervus version 2.0 (Marshall *et al*. 1998). The conformance to Hardy-Weinberg expectations of each locus was calculated using GENEPOP version 3.4 (Raymond & Rousset 1995). When corrected for multiple comparisons using the Bonferroni method.

RstCalc (Goodman 1999) and GENEPOP were used to calculate R_{st} (Slatkin 1995) and F_{st} (Wright 1969) of *T. viridipunctatus* subpopulations, *T. luyeanus* subpopulations, and both sides of the river-bank populations. In addition, calculations of correlation between genetic and geographic distance were performed through, R_{st} and geographic distance, respectively.

Intraspecific and interspecific migration rates of *T. viridipunctatus* and *T. luyeanus* were calculated by BayesAss (Wilson and Rannala 2003).

Analysis 2: The Bayesian clustering method described by Pritchard *et al*. (2000) and implemented in the program STRUCTURE 2.1 were applied to the genetic data. Using multilocus genotypes to assign individuals to K number of groups. To choose the 100,000 burn-in length and to 1,000,000 iterations, several runs were assayed at each number of groups. Posterior probabilities of the number K were estimated assuming uniform prior values on K and comparing the Ln of the probability of the data for each one. The highest likelihood value is assumed to indicate the number of groups in sample data.

After the number of K groups was estimated and compared to the Ln of the probability of the data, the highest likelihood value was assumed to indicate the number of groups in the data pool. However, if the K values were similar, Kruskal-Wallis, Mann-Whitney-Wilcoxon, and likelihood

ratio tests were used to calculate the differences of likelihood, between the two K values. If there were no significant statistic differences between them, the K value with the more simple and realistic value in accordance with taxonomy of the genus *Takydromus* was chosen.

Analysis 3: Using STRUCTURE to assign individuals to the distinct K groups with (1) all 13 loci and (2) 6 loci which show high R_{st} values ($R_{st} > 0.1$). Integrating and comparing data from (1) and (2) helps us evaluate the results as well as evaluate the effect of homoplasy on loci with low R_{st}.

Determination of hybrids

Mitochondrial DNA and microsatellite data were used to determine individuals which may belong one of the four situations below (Figure 2):

(1) Pure species: Individuals with both mitochondrial DNA and microsatellite loci congruent with geographic distribution.

(2) Migrants: Individuals with both mitochondrial DNA and microsatellite loci incongruent with geographic distribution were assigned to migrants.

(3) Hybrids: Individuals with mitochondrial DNA congruent with geographic distribution, but representing partial microsatellite characteristics of the other species (>10%, the range of variance of Ln estimated by STRUCTURE).

(4) Hybrids or descendants of hybrids: Individuals with mitochondrial DNA incongruent, but microsatellite pattern congruent or partially congruent with geographic distribution. Descendants of hybrids may have experienced recent or ancestral female introgression unless

previously assigned to migrants.