Zoological Studies **45**(3): 285-297 (2006)

Zoological Studies

The Phylogeography and Population Demographics of Selected Freshwater Fishes in Taiwan

Chyng-Shyan Tzeng[1,*], Yeong-Shin Lin[1,2], Si-Min Lin[1,3], Tzi-Yuan Wang[1,4], and Feng-Yung Wang[1]

[1]*Department of Life Science, National Tsing Hua University, Hsinchu, Taiwan 300, R.O.C.*

[2]*Present address: Institute of Biological Science and Technology, National Chiao Tung University, Hsinchu, Taiwan 300, R.O.C.*

[3]*Present address: Department of Life Science, Chinese Culture University, Taipei, Taiwan 111, R.O.C.*

[4]*Present address: Genomics Research Center, Academia Sinica, Taipei, Taiwan 115, R.O.C.*

(Accepted September 22, 2005)

Chyng-Shyan Tzeng, Yeong-Shin Lin, Si-Min Lin, Tzi-Yuan Wang, and Feng-Yung Wang (2006) The phylogeography and population demographics of selected freshwater fishes in Taiwan. *Zoological Studies* **45**(3): 285-297. Unlike most fluvial species, which are usually isolated by geographical barriers, low-elevation wetland fishes have more opportunities to exchange migrants (i.e., higher gene flow). Our phylogeographic analyses revealed that Taiwanese populations of the ricefish (*Oryzias latipes*), paradise fish (*Macropodus opercularis*), and a species of the landlocked freshwater goby (*Rhinogobius giurinus*) are all paraphyletic and clustered with Chinese populations. The 3 ricefish populations known from Taiwan may have been derived from multiple invasion events. We hypothesized that the original ricefish populations distributed throughout northwestern Taiwan significantly declined from their overall range, and the surviving inhabitants were restricted to Shuanglien Pond. This population thereby exhibited high genetic diversity. Alternatively, humans probably unintentionally introduced the other 2 populations. We also found that the abundant freshwater goby and the endangered paradise fish populations in Taiwan had both recently expanded from China. The separation time is insufficient for the Taiwanese populations to have diverged and developed elevated genetic diversity. In other words, the actual genetic homogeneity of paradise fish in Taiwan is not necessarily derived from a recent extinction. The other example of shallow differentiation among Taiwanese fishes is the Formosan masu salmon, *Oncorhynchus masou formosanus*. Its low genetic diversity may partly have been the result of a founder effect when initial masu salmon populations colonized Taiwan during the last glaciation.
http://zoolstud.sinica.edu.tw/Journals/45.3/285.pdf

Key words: Biogeography, Evolution, Mutation rate, Mitochondrial control region, Conservation.

Freshwater fishes are usually appropriate for use in biogeography studies, primarily because of their restricted distribution and the strong association between evolutionary history and geological events. Unfortunately, their native habitats have been seriously altered by human activities in recent years. Several local extinctions have occurred, especially among lowland species in Taiwan.

The typical example is the ricefish, *Oryzias latipes* (Temminck and Schlegel) (Cyprinodontiformes: Adrianichthyidae), which is a small fish inhabiting ponds, brooks, and even rice paddies. Sexually mature females attach fertilized eggs to the forepart of their anal fins, and then deposit them under aquatic vegetation. Although *O. latipes* is common in China and Japan, this species was thought to be extinct in Taiwan for more than 40 yrs until the rediscovery of the Shuanglien Pond population in northeastern Taiwan in 1992 (Lin 1995). Two other isolated populations were subsequentially discovered in nearby areas.

The phylogeography of such wetland species may differ from that of fluvial species because of differences in connectivity among populations or

* To whom correspondence and reprint requests should be addressed. Tel and Fax: 886-3-5742765.
E-mail:cstzeng@life.nthu.edu.tw

regional groups, since there is a lack of significant isolation between regional inhabitants. Previous phylogeographic studies of freshwater fishes in Taiwan have concentrated only on fluvial species (Wang et al. 1999, Wang et al. 2000). Therefore, phylogenetic analysis of such species will be important for both biogeography and conservation biology.

In addition to ricefish, we compared 2 other endangered species in Taiwan. One is the paradise fish (*Macropodus opercularis* (Linnaeus), Perciformes: Osphronemidae), which thrives in habitats analogous to those of the ricefish. This species is well known for the bubble nest built by the male to house and protect fertilized eggs and the resultant fry. The other is the fluvial landlocked Formosan masu salmon (*Oncorhynchus masou formosanus* (Jordan and Oshima), Salmoniformes: Salmonidae), the southernmost population of Pacific salmon. With a remaining size recently found to be approximately 3000 individuals (Tzeng and Yang 2003), it is paramount to learn the genetic structure of this seriously endangered species in order to promote its conservation. For comparison, we included respective sister groups of both the paradise fish and masu salmon, *Macropodus chinensis* (Bloch) and *Oncorhynchus masou masou* (Brevoort).

In contrast to the previously mentioned species, not all wetland species have undergone local extinctions. The goby genus *Rhinogobius* (Perciformes: Gobiidae) contains at least 40 species distributed among Western Pacific drainages (Chen et al. 1998). The taxonomic status of this genus in Taiwan was revised by Chen (1994) using both molecular and morphological characters. One of the most abundant species in this genus is *Rhinogobius giurinus* (Rutter), a landlocked freshwater goby distributed extensively in most low-elevation habitats in eastern Asia. These fish dig breeding cavities under stones where they deposit thousands of eggs and produce planktonic larvae that may enhance gene flow during floods.

Since both endangered and abundant species were considered, we were able to compare their genetic diversities, which is usually used to reflect the potential risk of a species facing extinction, especially in endangered populations (see O'Brien 1994, Frankham 1999, Cox and Hebert 2001, Templeton et al. 2001, Uphyrkina et al. 2002), because genetic diversity is determined by the specific evolution rate and effective population size (Kimura 1969, Watterson 1975). Comparing a population's size with its genetic diversity may reveal how equilibrium was reached, and help determine the effects of demographics on genetic diversity. In this study, we investigate and discuss the biogeography, evolution, conservation, and recent history of selected Taiwanese fish populations.

MATERIALS AND METHODS

DNA isolation

In total, 116 specimens from 9 species and subspecies (Table 1) were sampled from Taiwan, China, and Japan (Fig. 1). The DNA samples were extracted from muscle or fin tissue (Kocher et al. 1989). Approximately 0.1 g of tissue was homogenized in 1 ml digestion buffer (10 mM Tris-HCl (pH 8.0) 2 mM EDTA, 10 mM NaCl, 1% SDS, 10 mg/ml DTT, and 0.5 mg/ml proteinase K) and incubated at 37°C for 8 to 16 h, followed by a standard phenol and chloroform extraction (Kocher et al. 1989). The DNA samples were preserved in 50 μl TE buffer.

PCR amplification and sequencing

PCR amplifications for the complete mitochondrial control region (D-loop) were performed in a mixture with a final volume of 50 μl containing 1 μl crude DNA, 200 μM of each dNTP, 0.3 μM of each primer, and 1 unit of *Super Taq* (HT Biotechnology Limited, Cambridge, UK); the buffer was supplied by the manufacturer. The PCR and sequencing primers are listed in table 2. Variable conditions modified according to Innis et al. (1989) were designed for amplification: at 93°C for 3 min, 93°C for 30 s, 40-55°C for 40 s, 72°C for 1 min (repeated 35-45 times); and 72°C for 10 min. Amplification was repeated 3 times. This method is to ensure quality control, such as avoiding random amplification errors. The product mixture was employed as the template for sequencing, which was performed using the Sequenase PCR Product Sequencing Kit (United States Biochemical, Cleveland, OH).

Phylogenetic and population genetic analyses

Sequences were aligned using ClustalW (Thompson et al. 1994) and then manually modified. Phylogenetic analyses were performed using the Neighbor-joining method (Saitou and Nei 1987) with Tamura-Nei (TN) distances (Tamura and Nei

1993) and 5000 bootstrap replications (Felsenstein 1985), as implemented in the MEGA package (Kumar et al. 1993). Alignment gaps were completely deleted.

Statistical values of populations among species including θ from average pairwise differences (π) and segregating sites (s), Tajima's D (1989), Fu's Fs (1997), and Fu and Li's D^* and F^* (1993) were also estimated using the DNASP package (Rozas and Rozas 1999). Statistical tests were only applied for populations with a sample size exceeding 10. *Rhinogobius giurinus* and *M. opercularis* in Taiwan were each considered as 1 population, because these low-elevation wetland fishes can easily migrate during ordinary floods and are not isolated by geographical barriers, which was supported by our phylogenetic results. These statistical tests of neutrality of mutations were used to detect a class of alternative models, under which DNA polymorphisms tend to exhibit excesses of rare alleles or young mutations. The mismatch distribution analysis was also performed using Arlequin ver. 2.000 (Schneider et al. 2000).

Table 1. Samples analyzed

Oryzias latipes sinensis

Accession no.[a]	Locality[b]	No.[c]
AF383179	Taiwan (d)	5
AF383180	Taiwan (d)	2
AF383181	Taiwan (d)	1
AF383182	Taiwan (d)	1
AF383183	Taiwan (d)	1
AF383184	Taiwan (d)	1
AF383185	Taiwan (d)	1
AF383186	Taiwan (d)	1
AF383187	Taiwan (d)	1
AF383188	Taiwan (d)	1
AF383189	Taiwan (d)	1
AF383190	Taiwan (d)	1
AF383191	Taiwan (b)	5
AF383192	China (G)	1
AF383193	China (G)	1
AF383194	China (L)	1
AF383195	Taiwan (e)	3

Oryzias latipes latipes

Accession no.	Locality	No.
AF383196	Japan (B)	2
AF383197	Japan (B)	1
AF383198	Japan (A)	3
AF383199	Japan (A)	1
AF383200	Japan (A)	1
AF383201	Japan (B)	1

Rhinogobius giurinus

Accession no.	Locality	No.
AF383202	Taiwan (u)	1
AF383203	Taiwan (a)	1
	Taiwan (p)	1
AF383204	Taiwan (j)	1
AF383205	Taiwan (j)	1
AF383206	Taiwan (j)	1
AF383207	Taiwan (j)	1
AF383208	Taiwan (i)	1
AF383209	Taiwan (f)	1
AF383210	Taiwan (a)	1
	Taiwan (c)	1
	Taiwan (q)	1
AF383211	Taiwan (k)	1
AF383212	Taiwan (l)	1
AF383213	Taiwan (r)	1
AF383214	Taiwan (t)	1
	Taiwan (v)	1
	Taiwan (w)	1
AF383215	China (M)	1
AF383216	China (F)	1
AF383217	China (G)	1
AF383218	China (N)	3
AF383219	China (M)	3
AF383220	China (K)	1
AF383221	China (I)	1
AF383222	China (H)	1

Rhinogobius candidianus

Accession no.	Locality	No.
AF383223	Taiwan (e)	1

Rhinogobius gigas

Accession no.	Locality	No.
AF383224	Taiwan (a)	1

Macropodus opercularis

Accession no.	Locality	No.
AF359366	Taiwan (g)	1
	Taiwan (h)	2
	Taiwan (s)	1
	Taiwan (x)	1
AF359367	Taiwan (m)	2
AF359368	Taiwan (o)	2
AF359369	Taiwan (o)	1
AF359370	Taiwan (s)	1
AF359377	China (K)	1
AF411909	China (J)	1
AY092029	China (O)	2
AY092030	China (O)	1
AY092031	China (O)	2

Macropodus chinensis

Accession no.	Locality	No.
AF359372	China (F)	1
AF359373	China (F)	1
AF359374	China (F)	1
AF359375	China (E)	1
AF359376	China (E)	1

Oncorhynchus masou formosanus

Accession no.	Locality	No.
AF425592	Taiwan (n)	11
AF425593	Taiwan (n)	1

Oncorhynchus masou masou

Accession no.	Locality	No.
AF429775	Japan (D)	1
AF429776	Japan (D)	1
AF429777	Japan (A)	2
AF429778	Japan (A)	3
AF429779	Japan (C)	1
AF429780	Japan (C)	4

[a]The accession number is deposited in GenBank. [b]Localities are shown in figure 1. [c]Sample size.

RESULTS

The mitochondrial control region and part of the neighboring genes of all 116 samples were amplified, sequenced, and deposited in GenBank (with 69 unique sequences) (Table 1). Since only the control region was considered in further analyses and the aligned gaps were eliminated in this study, some sequences with their own unique accession numbers have the same genotype in the phylogenetic trees (Fig. 2). The ricefish has at least 5-7 tandem repeat segments (5'-TGCATGCGCGT-3' on the light strand) in the beginning of the control region gene. Table 3 lists the lengths of the control regions after eliminating extra repeats and alignment gaps.

The phylogenetic tree revealed that the ricefish population in Taiwan clusters with Chinese populations, which suggests that they belong to the same subspecies, *Oryzias latipes sinensis* Chen, Uwa and Chu. The karyotype analysis also supports this discovery (Lin et al. 1999). Unexpectedly, the 3 populations surveyed in Taiwan are not clustered together as a monophyletic clade; rather they were grouped within distinct clades exhibiting high bootstrap values (Fig. 2).

A monophyletic clade of the *Rhinogobius giurinus* population in Taiwan does not exist either. The clustering of 3 Chinese specimens (from the F, K, and M populations) and the Taiwanese populations are supported by a bootstrap value of 64. We defined this association as clade R-I, and the other as clade R-II which includes the remaining

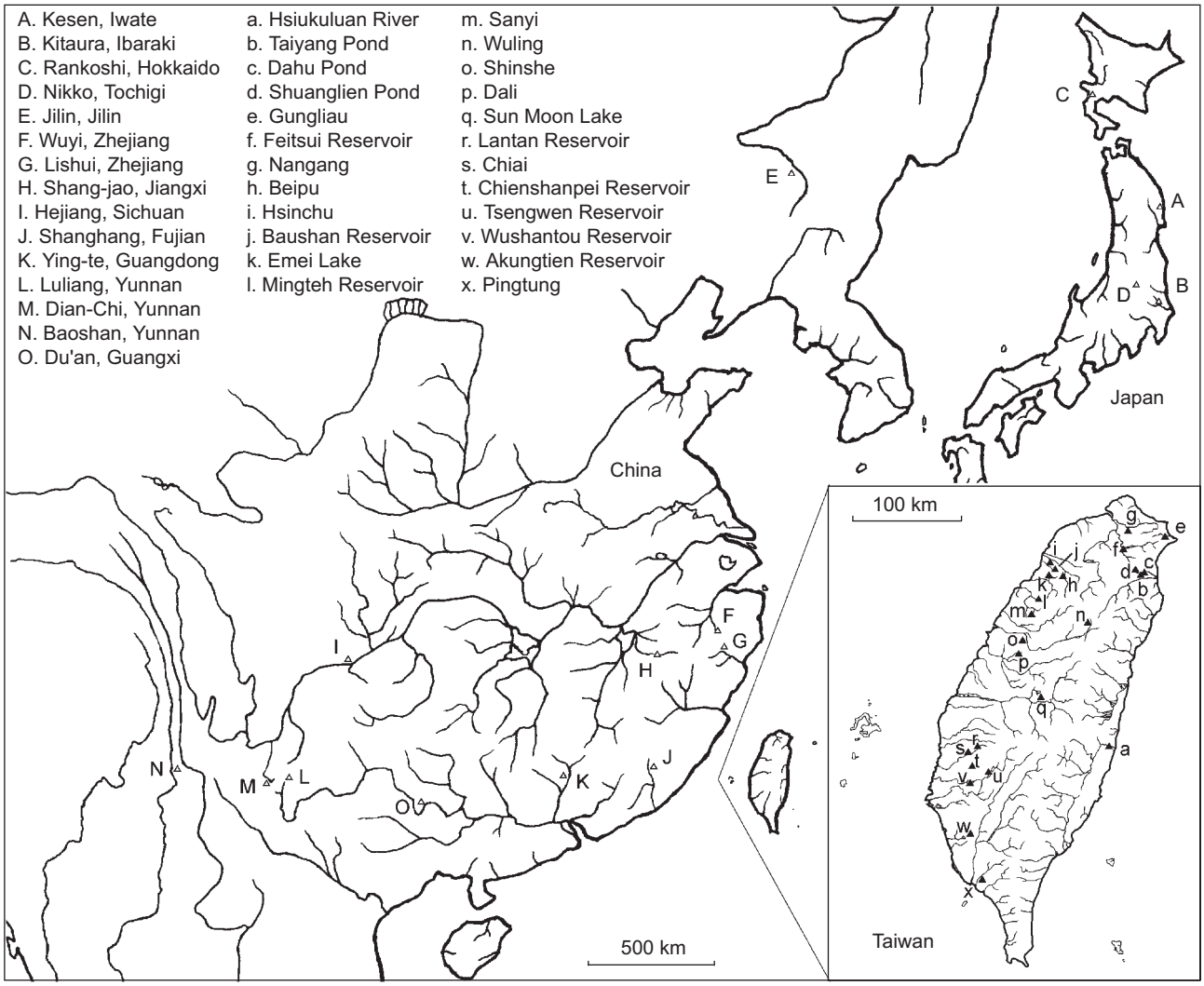

Fig. 1. Sampling localities indicated by small letters and solid triangles in Taiwan and by capital letters and hollow triangles in China and Japan, respectively.

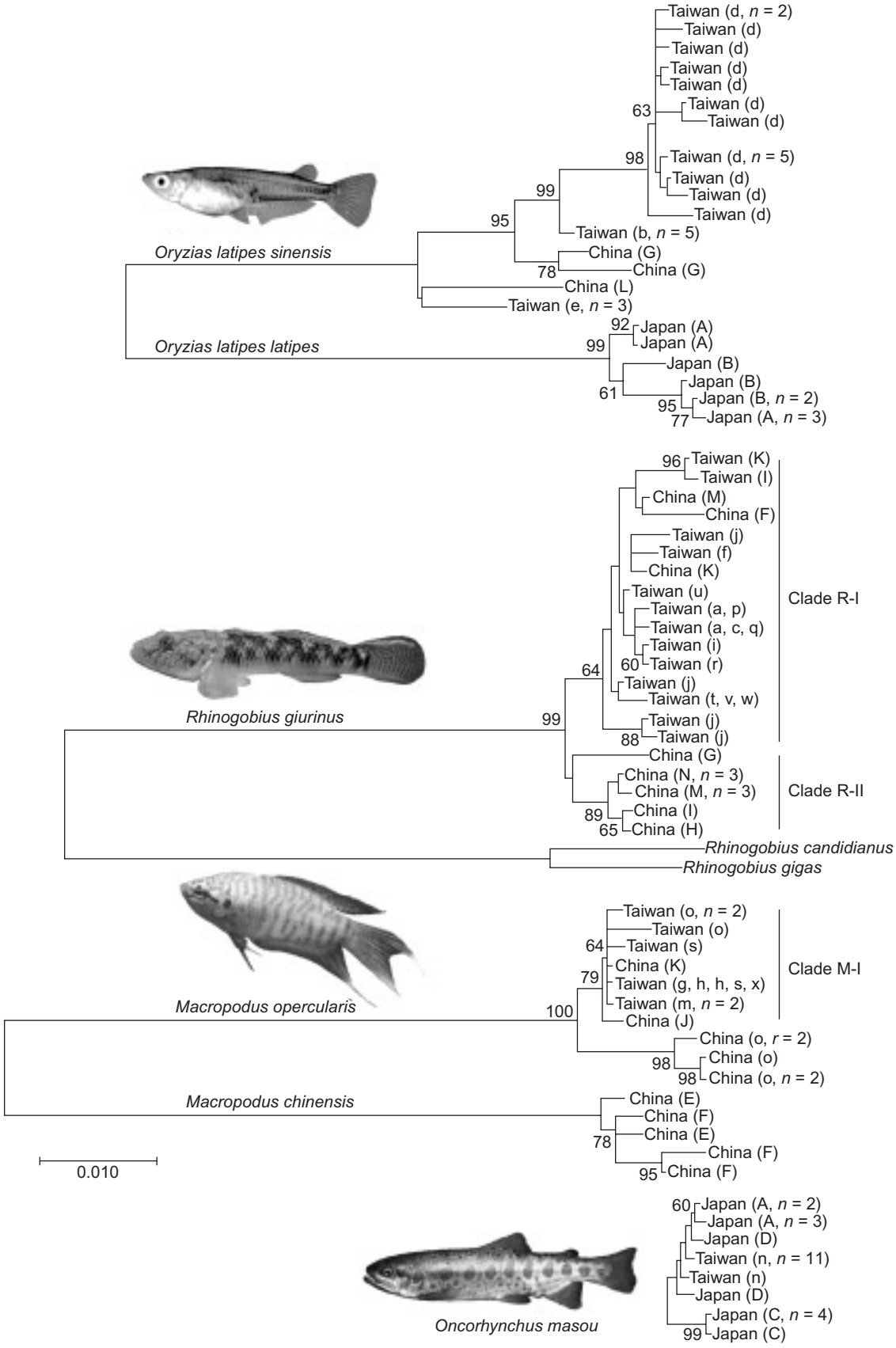

Fig. 2. Phylogenetic trees constructed using the Neighbor-joining method (Saitou and Nei, 1987) with Tamura-Nei (TN) distances (Tamura and Nei, 1993) with 5000 bootstrap replications (Felsenstein, 1985). Only bootstrap values larger than 60 are displayed. The scale bar is corrected distahce (TN distance = 0.010).

Chinese specimens.

The other 2 *Rhinogobius* species in this study, *R. candidianus* (Regan) and *R. gigas* Aonuma and Chen, are from western and eastern Taiwan, respectively, and are allopatrically separated by the Central Mountain Range (CMR). They are distinct by 2.58% (sequence divergence assuming the Tamura-Nei model), which can be used for estimation of the mutation rate.

Similarly, the Taiwanese population of *M. opercularis* is also clustered with Chinese specimens (defined as clade M-I). We can recognize that the Taiwanese populations of these 3 wetland species are all paraphyletic, whereas most of the stream species in Taiwan are endemic or represent monophyletic populations (Wang et al. 1999, Wang et al. 2000, Poh 2001).

Formosan masu salmon can be differentiated from the Japanese subspecies by having fewer vertebrae (61.56 vs. 64.24-64.45), dorsal rays (13.83 vs. 15.37-15.40), anal rays (12.61 vs. 14.20-14.80), pectoral rays (13.12 vs. 14.60-14.80), pyloric ceca (29.10 vs. 35.20-39.22), and black spots on the back (21.88 vs. 78.13) (Yasue 1982, Watanabe et al. 1985). Surprisingly, these 2 subspecies could not be distinguished by their mitochondrial control region sequences. Eleven of 12 examined Formosan masu salmon specimens have identical sequences, while the other specimen has only 1 transition substitution. No significant clustering could be recognized for the masu salmon.

Table 3 lists the statistical values estimated using the DNASP package (Rozas and Rozas

Table 2. Primers used

Position	Chain	Sequence	Genus to which applied
Cytochrome *b*	L	5'-ATYCTACGRTCAATYCC-3'	*Macropodus*
Cytochrome *b*	L	5'-ATTATCCTAACATGAATTGG-3'	*Oryzias*
Cytochrome *b*	L	5'-CCTAACATGAATTGGAGG-3'	*Oryzias*
Thr-tRNA	L	5'-GCTCAATTGCAAGAGCGCCGG-3'	*Oryzias*
Pro-tRNA	L	5'-CCTTGTGCTCAGAGAAAGGGG-3'	*Oryzias*
Pro-tRNA	L	5'-AACTTCCATCCTCAACTCCCAAAGC-3'	*Rhinogobius*, *Macropodus*, *Oncorhynchus*
Control region	L	5'-CCTGAAAACCCCCCGGAAAC-3'	*Oryzias*
Control region	L	5'-GCCCTAAAATGGTCGAGTACGAGC-3'	*Oryzias*
Control region	L	5'-TTTCACAGTGCATACAGACC-3'	*Oryzias*
Control region	L	5'-TTGCATAACCCAAAACGGCC-3'	*Oryzias*
Control region	H	5'-ATATCTTACAATAATTCACC-3'	*Oryzias*
Control region	H	5'-AAAAAGGGAACCAGCTGCCC-3'	*Oryzias*
Control region	H	5'-ATTGACTTGCATGGACGAGG-3'	*Oryzias*
Control region	H	5'-GCATGCAGGTACGGCGGACC-3'	*Oryzias*
Control region	H	5'-ATGCACGTGTTGGGGGCCGG-3'	*Oryzias*
Control region	L	5'-GATGCGTTATCGGTTAACGAAGG-3'	*Rhinogobius*
Control region	L	5'-CACAGTGCAGCGCTAAGGC-3'	*Rhinogobius*
Control region	H	5'-CTTGAAGAGAAGGTGAGTAATGG-3'	*Rhinogobius*
Control region	H	5'-CCATTAGAGTGAACGCCCGGC-3'	*Rhinogobius*
Control region	H	5'-CTTCTAATAAAATCTTTTCACC-3'	*Rhinogobius*
Control region	L	5'-TAATAATTATWCAGGAC-3'	*Macropodus*
Control region	L	5'-CTATTACTGGCATCTGG-3'	*Macropodus*
Control region	H	5'-TGKWCCTGAAATAGGAACC-3'	*Macropodus*
Control region	H	5'-TYYTAGGAGTTTAGGGGG-3'	*Macropodus*
Control region	L	5'-CACGTGATAATAACC-3'	*Oncorhynchus*
Control region	L	5'-GGGCACGAGGAGCCG-3'	*Oncorhynchus*
Control region	L	5'-CTTTTGATGTACTTTA-3'	*Oncorhynchus*
Control region	L	5'-GGAGAACCCTATGCAT-3'	*Oncorhynchus*
Control region	H	5'-GGCTGCCTGGGCGTG-3'	*Oncorhynchus*
Control region	H	5'-CGGAGCCCGTGTTAGC-3'	*Oncorhynchus*
Phe-tRNA	H	5'-GCTTTAATTAAGCTACG-3'	*Macropodus*
Phe-tRNA	H	5'-ATCTTAACATCTTCAGTG-3'	*Oryzias*
12S rRNA	H	5'-GGGCATTCTCACGGGGATCGG-3'	*Oncorhynchus*
12S rRNA	H	5'-GGGCATTCTCACGGGGATGCG-3'	*Oryzias*, *Rhinogobius*, *Macropodus*

1999) for the major populations. Only Fu's *Fs* and Tajima's *D* tests for the Shuanglien Pond *O. latipes* population, and Fu's *Fs* test for the Taiwanese *R. giurinus* population are significant. Except for the Formosan masu salmon, for which only 1 substitution is recognized, the mismatch distributions are represented in figure 3. A pronounced wave distribution is recognized for *O. latipes* and *R. giurinus*, while *M. opercularis* displays a smooth decline.

DISCUSSION

Mutation rate

The mutation rate of *Rhinogobius* gobies was calculated by comparing the genetic distance between *R. candidianus* and *R. gigas*. These 2 species, previously proposed as being sister taxa (Chen 1994), are allopatrically distributed in western and eastern regions of Taiwan.

Poh (2001) built a molecular clock based on the cytochrome *b* gene following (Lin et al. 2001). The author suggests that *Onychostoma barbatulus* (Pellegrin) (Cypriniformes: Cyprinidae) in Taiwan is 4.90 million yrs old (Ma), corresponding with the formation age of Taiwan (5 Ma, Teng 1990, Huang et al. 1995, Huang et al. 1997). In the same study, the TN distance for the control region at the same node was calculated to be 2.84%. Therefore, the estimated mutation rate of the mitochondrial control region for *O. barbatulus* is 0.29% per million years (My).

Similar to *R. candidianus* and *R. gigas*, 2 *O. barbatulus* populations are also isolated by the northern CMR. They likely diverged since 2.62 Ma as determined by applying the TN distance of 1.54% and the mutation rate of 0.29%/My. Assuming that the speciation event of the 2 sister gobies occurred at the same time with the rise of the CMR, the mutation rate for the genus *Rhinogobius* would be estimated as 0.49%/My using the TN distance of 2.58% and a divergence time of 2.62 Ma.

The average TN distance for the control region between Chinese and Japanese subspecies of *O. latipes* was also calculated to be 9.84%. The separation between Japan and mainland Asia, which occurred approximately 15 Ma (Taira et al. 1989), correlates with the divergence time of these 2 subspecies. The mutation rate of the mitochondrial control region was therefore estimated to be 0.33%/My (TN distance), which is approximately -33% slower than the rate for *Rhinogobius*. Although we used the TN distance to correct it, the value is probably underestimated considering the saturation problem (e.g., Arbogast et al. 2002), especially when among-site rate variations exist (e.g., Yang 1994, Arbogast and Slowinski 1998).

Few studies have tried to estimate the divergence rate for fish mitochondrial control regions because of insufficient fossil records. Donaldson and Wilson (1999) reported a fast mutation rate of 1.8%/My (*p* distance, including insertions and deletions; 3.6% for divergence rate) for *Centropomus* fishes based on the rate of the cichlid's protein-coding ND2 gene proposed by Kocher and Carleton (1997). Martin and Palumbi (1993) proposed a much-slower divergence rate, ranging from 0.25% to 0.45%/My for salmon, compared to the Perciformes. Bagley and Gall (1998) suggested that the mutation rate of rainbow trout ranges from 0.4% (viz. McKay et al. 1996) to 1.0%/My (viz. Shedlock et al. 1992, which is based on mammalian results). Oohara et al. (1997) estimated the origin of the genus *Oncorhynchus* by comparing fossil records. They estimated that this genus appeared at between 11 and 30 Ma when using Martin and Palumbi's rate (1993) rather than 5 Ma using Shedlock et al.'s rate (1992). By referring to our results, it is reasonable to conclude that 0.25%-0.45%/My is an acceptable range for the mutation rate of fish mitochondrial control regions, although the equivalent for the Perciformes or cichlids may be faster.

Phylogeography

Oryzias latipes in Taiwan is threatened by habitat loss and environmental pollution. Since recorded by Oshima (1919), the distribution of this fish has been reduced from the overall northwestern region of Taiwan to limited and fragmented areas. With only 3 spatially restricted populations discovered in the current survey, it is difficult to reconstruct the phylogeography of the ricefish. However, we propose the following 3 alternate possibilities to explain their current genetic pattern.

The 1st hypothesis is that the ricefish in Taiwan was widespread as a unique panmictic population and exhibited great genetic diversity with frequent gene flow over its history. Eventually, large-scale extinctions occurred and the resulting bottleneck created 3 distinct and isolated populations. If this is true, the resulting populations should either maintain some of their ancient haplotypes, or be completely purified and

fixed to 1 type.

Under this hypothesis, the intra-population genetic diversity of population d would be difficult to explain. Long-term isolation between population d and the other populations would have been necessary to build up genetic diversity after the bottleneck purification. According to the mutation rate of mitochondrial control region mentioned above (of 0.29%/My) and the largest divergence discovered between specimens (0.87%, by the TN distance), the isolation event should have been maintained for at least 1.5 My.

Population d is located in Shuanglien Pond (Fig. 1), 18 ha in size and 466 m above sea level. A slumping event created this pond, and only a waterfall connects this pond with a stream, thus presenting a physical barrier for this wetland species. However, since this pond formed approximately 5000 (Liew 1994) to 50,000 (Hsu et al. 2005) yrs ago, this hypothesis (an isolation event having been maintained for 1.5 My) is refuted considering the age of the pond.

The 2nd hypothesis is that diversity was derived not from a large population but from subdivided populations isolated by geographical barriers. However, this hypothesis is not supported by the geographical and geological evidence. The present locality of population b is downstream from Shuanglien Pond at a distance less than 6 km. Although the barrier likely exists at the present time, 50,000 yrs is still not a sufficient length of time as previously indicated.

Finally, the 3rd hypothesis suggests that the 2 lowland Taiwan populations (b and e) recognized today were secondary colonizers in comparatively recent years. *Oryzias latipes* could have colonized Taiwan from China around 5 Ma, while population d, representing the original Taiwanese haplotypes, could have been maintained for at least 1.5 My. The gene flow between Taiwanese populations was not restricted due to weak geographical isolation until a remnant was possibly isolated in the slumping pond. Conversely, populations b and e expanded from China more recently.

Since ricefish attach their fertilized eggs to aquatic weeds, Chinese settlers may have imported some ricefish along with other fishes for aquaculture, such as the 4 Chinese carp species (*Mylopharyngodon piceus*, *Aristichthys mobilis*, *Hypophthalmichthys molitrix*, and *Ctenopharyngodon idellus*) imported to Taiwan several 100 yrs ago (Tzeng 1986). Either a founder effect or a significant population decline would have eliminated the genetic diversity of these transported populations. Another likely opportunity for such a secondary invasion occurred during the latest glaciation, which created a land bridge between Taiwan and the Asian mainland approximately 10,000 to 100,000 yrs before the present (Emery et al. 1971). However, the absence of these foreign genotypes in population d diminishes this possibility. Although the actual circumstances may have been more complicated, the 3rd hypothesis is the most parsimonious with the data we possess.

The phylogeny of *R. giurinus* haplotypes illustrates that clade R-I contains all Taiwanese specimens and 3 Chinese specimens from sites F, K, and M (Fig. 2). The TN distance of this clade is 1.5%, implying a divergence estimated to have occurred approximately 1.5 Ma when applying the mutation rate of 0.49%/My.

If this clade had been created with the formation of Taiwan, it would be difficult to explain why 25% of the Chinese specimens analyzed carried clade R-I haplotypes. There could potentially have been an elevated amount of gene flow during glaciations. Human distribution of the fish populations during the planktonic larval stage may also have occurred. However, this assumption raises another concern: the absence of clade R-II haplotypes in Taiwan. Gene flow during the glaciations should have been bi-directional; however, more migrants are from the mainland than from the island based on the model of island-mainland gene flow. Transportation of fish by human activities from China to Taiwan would also have been much more frequent than in the other direction.

Incomplete lineage sorting is the most reasonable explanation in this case. We speculate that clade R-I was originally a large population inhabiting southeastern China. A part of this population recently migrated to Taiwan, and maintained most of the haplotypes as a result of insufficient time for genetic drift and accumulation of mutations.

It should be emphasized that several events resulting in gene flow may have occurred in the past, but only the last event could be detected. The substitution number of the entire control region (of about 840 bp) for 100,000 yrs in the mutation rate (0.49%/My) would be approximately 0.41. This small number would be difficult to identify, unless a large number of specimens from Taiwan and China were analyzed. Since the timing of the migration of 100,000 yrs (during the glaciations) or 100 yrs ago (by human activities) is undistinguishable, both natural and artificial movements should be considered.

Although paradise fish are almost as rare as ricefish in Taiwan presently, the topology of their phylogenetic tree is rather similar to that of the abundant goby, *R. giurinus* (Fig. 2). This implies a similarity in the migratory patterns between *M. opercularis* and *R. giurinus*. It is notable that the low genetic divergence and the deficiency of genetic diversity in the paradise fish occurred not only in the Taiwanese population, but also in the entire clade M-I. This phenomenon indicates that the genetic homogenization of paradise fish in Taiwan was not necessarily derived from current population dynamics. A recent expansion of clade M-I from a small population would have had significant effects as well.

We suggest a similar chain of events for the Formosan landlocked masu salmon, *O. masou formosanus*, of Taiwan. When comparing it with the Japanese populations, the amount of genetic diversity of the Taiwanese population is so small that population decline or a founder effect may have had a greater impact on Formosan masu salmon. Numachi et al. (1990) proposed that Formosan masu salmon evolved from 100,000 to 800,000 yrs ago. The shortest genetic distance between Taiwanese and Japanese populations is 0.1%. Applying the mutation rate of 0.25% to 0.45%/My, 110,000 to 200,000 yrs ago could be suggested as the upper boundary of their divergence time, which corresponds to the last glaciation (Emery et al. 1971).

Comparison of genetic diversity and population demographics

The abundant goby and the endangered ricefish in Taiwan were found to have incongruous genetic diversities. The value of $\theta(\pi)$ of the Taiwanese *R. giurinus* population is 1.89 times larger than that of *O. latipes* population d (Table 3). It was also calculated to be 1.17 for $\theta(s)$.

Using an infinite site model for an autosomal locus, Kimura (1969) and Watterson (1975) showed that genetic diversity is demonstrated by the equation $\theta = 4N_e u$, where N_e is the effective population size and u is the mutation rate, and the population is at equilibrium with selectively neutral mutations. For haploid mitochondrial DNA, $\theta = 2N_e u$.

Considering the current distribution in Taiwan and the mutation rate mentioned previously, our results indicate that these 2 species with extremely different values of N have surprisingly similar values for $\theta/2u$. If population subdivision of the goby contributed to its genetic diversity, the conflict would be more significant. Similar patterns from different organisms are also recognized (e.g., Pestano et al. 2000, Zawko et al. 2001, Bonnin et al. 2002, Balakrishnan et al. 2003).

This conflict cannot only be attributed to the small ratio of N_e/N as discovered in the northern pike (Miller and Kapuscinski 1997). According to our phylogenetic analyses, we suggest that the genetic diversity of *O. latipes* population d represents the original entire Taiwanese population, which provides a possible explanation of why this small population embraces such high diversity. The other explanation is the exponential population growth of *R. giurinus*, while its genetic diversity has not yet reached equilibrium.

Tajima (1989) proposed the statistical value, *D*, found by comparing the difference between $\theta(\pi)$ and $\theta(s)$. A positive value of *D* indicates possible balancing selection or population subdivision. A negative value suggests recent directional selection, a population bottleneck, or a purifying selection on deleterious alleles.

Fu (1997) further compared statistical tests including Tajima's *D*, Fu's *Fs*, and Fu and Li's *D** and *F** under population growth. When an

Table 3. Statistical values of control region sequences for populations among species. An asterisk(*) indicates statistical significance at $p < 0.05$

	n[a]	bps[b]	$\theta(\pi)$[c]	*s*[d]	$\theta(s)$[e]	Fu's *Fs*	Tajima's *D*	Fu and Li's *D**	Fu and Li's *F**
Oryzias latipes sinensis Taiwan (d)	17	936	2.507	16	4.733	-7.552*	-1.823*	-1.810	-2.095
Rhinogobius giurinus Taiwan	18	841	4.726	19	5.524	-4.901*	-0.561	0.494	0.221
Macropodus opercularis Taiwan	11	833	1.055	5	1.707	-0.627	-1.465	-1.445	-1.634
Oncorhynchus masou formosanus Taiwan (*n*)	12	1009	0.167	1	0.331	-0.476	-1.140	-1.330	-1.443

[a]Sample size. [b]Base pairs. [c]Genetic diversity calculated from average pairwise differences. [d]Segregating sites. [e]Genetic diversity calculated from segregating sites.

increasing population differs significantly from the initial size before it reaches a steady size, Fu's *Fs* test is the most powerful demonstration against logistic population growth, while the other 3 tests are less significant. Therefore, when only Fu's *Fs* test is significant, a growing population size, rather than non-neutral mutations, is implied as a cause.

The mismatch distribution also provides additional evidence for an exponential growth pattern when a pronounced wave is represented, while a smooth decline indicates an equilibrium status (Slatkin and Hudson 1991, Rogers and Harpending 1992).

Our results indicate that both the statistical tests (Table 3) and mismatch distribution (Fig. 3) support the expansion hypothesis for *R. giurinus* and reject the possibility of population subdivision. A departure from neutral expectations was also detected for the Shuanglien Pond ricefish population. Considering its genetic divergence, a founder effect followed by an expansion since they colonized Taiwan 1.5 Ma is a better explanation rather than a bottleneck effect generated by the slumping event of Shuanglien Pond 5000-50,000 yrs ago. This also implies that the slumping event did not actually cause the geographical isolation, and the population collapse did not occur until very recently, or an overrepresentation of sequence identity (no difference) would have been detected.

By contrast, the neutrality tests were not rejected for the paradise fish or masu salmon. Meanwhile, an equilibrium status was also implied for paradise fish according to its mismatch distribution. A small equilibrium population size or a bottleneck effect was suggested for the salmon. However, considering the similar phylogenetic tree topologies between *M. opercularis* and *R. giurinus*, and the original wide distribution of the paradise fish, we speculate that it is also possible that the expansion of *M. opercularis* occurred more recently and is not reflected by its genetic structure.

Conclusions

While fluvial species have limited opportunities for gene flow, due to significant geological events as river formation or glaciation, low-elevation wetland fishes can easily migrate during ordinary floods. The consequence is that stream populations usually have unique haplotypes, and their phylogenetic trees usually have deep branches and reflect geological events. By contrast, wetland fishes like *R. giurinus* and *M. opercularis* can expand widely in a short period of time, and thus

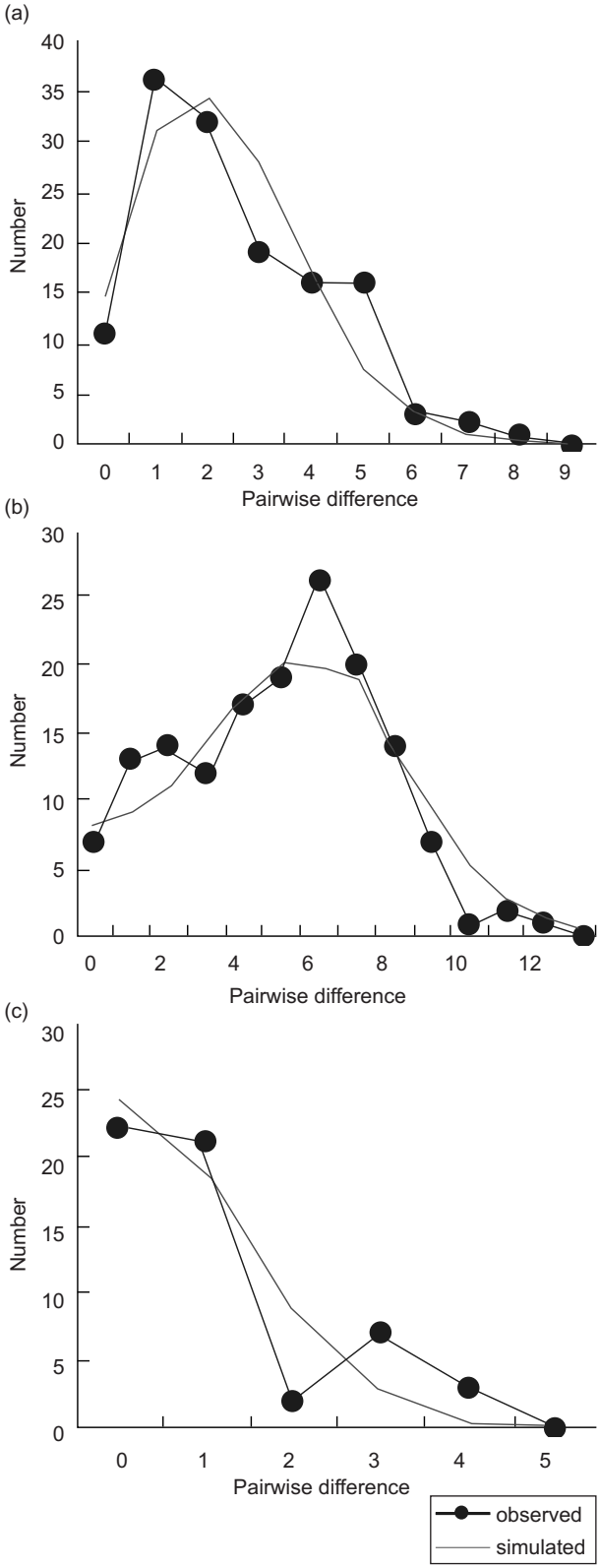

Fig. 3. Mismatch distributions for (a) the Taiwanese population d of *Oryzias latipes sinensis* (*n* = 17); (b) the Taiwanese population of *Rhinogobius giurinus* (*n* = 18); and (c) the Taiwanese population of *Macropodus opercularis* (*n* = 11).

express shallow divergence and low diversity.

Most fluvial fishes in Taiwan may have arrived with the formation of the island, and thus they exhibit endemic or monophyletic patterns (Wang et al. 1999, Wang et al. 2000, Poh 2001). One exception is the Formosan masu salmon, which used to be anadromous during the last glaciation until it was landlocked and isolated from Japanese populations.

Unfortunately, although wetland fishes can expand quickly, these low-elevation environments have also quickly disappeared in the past century. Under these circumstances, using genetic diversity to determine which population to protect may have some flaws. For example, while ricefish once experienced huge population increases, it is currently limited in restricted habitats in Taiwan. This is the reason we still can find substantial genetic diversity for the small ricefish population in Shuanglien Pond, which has recently been designated a wildlife sanctuary. However, if only this habitat is maintained and protected, genetic homogenization will eventually be derived from the insufficient population size. Human activities have depleted the habitable environments for ricefish, but the habitats for *R. giurinus* have since expanded with the construction of irrigation projects, enhancing its population growth. Even though the goby and ricefish have similar genetic diversities, similar conservation values are never implied.

Another example is the deficiency of genetic diversity in the paradise fish and masu salmon. It is inappropriate to judge if this species will become extinct using this index, because the deficiency is not necessarily correlated with recent population declines. The expansion event of paradise fish followed by these extinctions and the small equilibrium population size or a founder event of masu salmon could possibly be more-acceptable explanations.

Based on our analyses and the fact that increasing human activity has rapidly destroyed or polluted many low-elevation wetland habitats in Taiwan, we suggest that the investigation of biodiversity for species in these areas should be accelerated, and more resources should also be invested. Considering the unique genetic structure for each species or each local population, not only genetic diversity, but also its evolutionary history should be studied. It is important to determine which populations still maintain high genetic diversity, and the reason why the diversity is maintained. In this way, we can have some idea about the conservation value of each ecological habitat, and how to protect its genetic diversity, which should be useful for establishing appropriate conservation strategies.

Acknowledgments: We are grateful to Wen-Shiang Chang and Jian-Ping Jan for providing specimens, and Te-Yu Liao and Sheng-Tsung Chen for assistance in sample collecting. We also thank Geoffrey Cohen, Chaolun Allen Chen, Yu-Ping Poh, and the anonymous reviewers for valuable comments. This research was financially supported by the National Science Council, Republic of China (NSC86-2311-B-002-028-B17 and NSC87-2311-B-002- 015-B17), and permission to collect samples was obtained from the Council of Agriculture, Executive Yuan, Taiwan.

REFERENCES

Arbogast BS, JB Slowinski. 1998. Pleistocene speciation and the mitochondrial DNA clock. Science **282:** 1955a.

Arbogast BS, SV Edwards, J Wakeley, P Beerli, JB Slowinski. 2002. Estimating divergence times from molecular data on phylogenetic and population genetic timescales. Annu. Rev. Ecol. Syst. **33:** 707-740.

Bagley MJ, GAE Gall. 1998. Mitochondrial and nuclear DNA sequence variability among populations of rainbow trout (*Oncorhynchus mykiss*). Mol. Ecol. **7:** 945-961.

Balakrishnan CN, SL Monfort, A Gaur, L Singh, MD Sorenson. 2003. Phylogeography and conservation genetics of Eld's deer (*Cervus eldi*). Mol. Ecol. **12:** 1-10.

Bonnin I, B Colas, C Bacles, AC Holl, F Hendoux, B Destine, F Viard. 2002. Population structure of an endangered species living in contrasted habitats: *Parnassia palustris* (Saxifragaceae). Mol. Ecol. **11:** 979-990.

Chen IS. 1994. The systematic studies of *Rhinogobius brunneus* complex from Taiwan. Master thesis, National Sun Yat-Sen University, Kaohsiung. (in Chinese with English abstract)

Chen IS, CH Hsu, CF Hui, KT Shao, PJ Miller, LS Fang. 1998. Sequence length and variation in the mitochondrial control region of two freshwater gobiid fishes belonging to *Rhinogobius* (Teleostei: Gobioidei). J. Fish Biol. **53:** 179-191.

Cox AJ, PD Hebert. 2001. Colonization, extinction, and phylogeographic patterning in a freshwater crustacean. Mol. Ecol. **10:** 371-386.

Donaldson KA, RR Jr Wilson. 1999. Amphi-panamic geminates of snook (Perciformes: Centropomidae) provide a calibration of the divergence rate in the mitochondrial DNA control region of fishes. Mol. Phylogenet. Evol. **13:** 208-213.

Emery KO, H Nino, B Sullivan. 1971. Post-Pleistocene levels of the East China Sea. *In* KK Turekian, ed. The Late Cenozoic Glacial Ages. New Haven, CT: Yale University Press, pp. 381-390.

Felsenstein J. 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution **39:** 783-791.

Frankham R. 1999. Quantitative genetics in conservation biology. Genet. Res. **74:** 237-244.

Fu YX. 1997. Statistical tests of neutrality of mutations against population growth, hitchhiking and background selection. Genetics **147:** 915-925.

Fu YX, WH Li. 1993. Statistical tests of neutrality of mutations. Genetics **133:** 693-709.

Hsu B, WL Wang, TQ Lee, SR Song. 2005. Based on the fossil diatom records reconstruct the paleoclimate changes from Yilan during the last 45 kyrs. Abstract of 2005 Annual Join Symposium of Animal Behavior and Ecology, Society of Stream and Society of Biology, Taiwan, pp. 34.

Huang CY, PM Liew, M Zhao, Z An, TC Chang, CM Kuo, MT Chen, CH Wang, LF Zheng. 1997. Deep sea and lake sediment records of the Southeast Asian paleomonsoons in the last 25,000 kyrs. Earth Planet. Sc. Lett. **146:** 59-72.

Huang CY, PB Yuan, SR Song, CW Lin, C Wang, MT Chen, CT Shyu, B Karp. 1995. Tectonics of short-lived intra-arc basins in arc-continent collisionterrane of the Costal Range, eastern Taiwan. Tectonics **14:** 19-38.

Innis MA, DH Gelfand, JJ Sninsky, TJ White. 1989. PCR protocols: a guide to methods and applications. San Diego, CA: Academic Press.

Kimura M. 1969. The rate of molecular evolution considered from the standpoint of population genetics. Proc. Natl. Acad. Sci. USA **63:** 1181-1188.

Kocher TD, KL Carleton. 1997. Base substitution in fish mitochondrial DNA: Patterns and rates. *In* TD Kocher, CA Stepien, eds. Molecular systematics of fishes. San Diego, CA: Academic Press, pp. 13-24.

Kocher TD, WK Thomas, A Meyer, SV Edwards, S Pääbo, FX Villablanca, AC Wilson. 1989. Dynamics of mitochondrial DNA evolution in animals: amplification and sequencing with conserved primers. Proc. Natl. Acad. Sci. USA **86:** 6196-6200.

Kumar S, K Tamura, M Nei. 1993. MEGA: Molecular Evolutionary Genetics Analysis, version 1.02. University Park, PA: Pennsylvania State Univ.

Liew PM. 1994. Palynological study of Quaternary lake deposits in Yuan-Shan Ilan. NSC82-0202-M002-113. Taipei, Taiwan: National Science Council. (in Chinese with English abstract)

Lin SM. 1995. Characterization of Medaka fish (*Oryzias latipes*) mtDNA D-loop. Master's thesis, National Tsing Hua Univ., Hsinchu, Taiwan. (in Chinese with English abstract)

Lin SM, YS Lin, CF Huang, CS Tzeng. 1999. The phylogenetic study on the endangered ricefish (*Oryzias latipes*) from Taiwan. Acta Zool. Taiwanica **10:** 75.

Lin YS, YP Poh, CS Tzeng. 2001. A phylogeny of freshwater eels inferred from mitochondrial genes. Mol. Phylogenet. Evol. **20:** 252-261.

McKay SJ, RH Devlin, MJ Smith. 1996. Phylogeny of Pacific salmon and trout based on growth hormone type 2 and mitochondrial NADH dehydrogenase subunit 3 DNA sequences. Can. J. Fish. Aquat. Sci. **53:** 1165-1176.

Miller LM, AR Kapuscinski. 1997. Historical analysis of genetic variation reveals low effective population size in a northern pike (*Esox lucius*) population. Genetics **147:** 1249-1258.

Numachi K, T Kobayashi, KH Chang, YS Lin. 1990. Genetic identification and differentiation of the Formosan landlocked salmon, *Oncorhynchus masou formosanus*, by restriction analysis of mitochondrial DNA. Bull. Inst. Zool. Acad. Sinica **29:** 61-72.

O'Brien SJ. 1994. A role for molecular genetics in biological conservation. Proc. Natl. Acad. Sci. USA **91:** 5748-5755.

Oohara I, K Sawano, T Okazaki. 1997. Mitochondrial DNA sequence analysis of the masu salmon – phylogeny in the genus *Oncorhynchus*. Mol. Phylogenet. Evol. **7:** 71-78.

Oshima M. 1919. Contribution to the study of the freshwater fishes of the island of Formosa. Ann. Carn. Mus. **12:**169-328.

Pestano J, RP Brown, F Rodriguez, A Moreno. 2000. Mitochondrial DNA control region diversity in the endangered blue chaffinch, *Fringilla teydea*. Mol. Ecol. **9:** 1421-1425.

Poh YP. 2001. The study of the influence by the process of Taiwan island formation to the population genetics structure of freshwater fishes in Taiwan. Master thesis, National Tsing Hua Univ., Hsinchu, Taiwan. (in Chinese with English abstract)

Rogers AR, H Harpending. 1992. Population growth makes waves in the distribution of pairwise genetic differences. Mol. Biol. Evol. **9:** 552-569.

Rozas J, R Rozas. 1999. DnaSP version 3: an integrated program for molecular population genetics and molecular evolution analysis. Bioinformatics **15:** 174-175.

Saitou N, M Nei. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. **4:** 406-425.

Schneider S, D Roessli, L Excoffier. 2000. Arlequin: a software for population genetics data analysis. Ver 2.000. Geneva: Genetics and Biometry Lab, Department of Anthropology, University of Geneva.

Shedlock AM, JD Parker, DA Crispin, TW Pietsch, GC Burmer. 1992. Evolution of the salmonid mitochondrial control region. Mol. Phylogenet. Evol. **1:** 179-192.

Slatkin M, RR Hudson. 1991. Pairwise comparisons of mitochondrial DNA sequences in stable and exponential growing populations. Genetics **129:** 555-562.

Taira A, H Tokuyama, W Soh. 1989. Accretion tectonics and evolution of Japan. *In* Z Ben-Avraham, ed. The evolution of the Pacific Ocean margins. Oxford, UK: Clarendon Press, pp. 100-123.

Tajima F. 1989. Statistical method for testing the neutral mutation hypothesis by DNA polymorphism. Genetics **123:** 585-595.

Tamura K, M Nei. 1993. Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. Mol. Biol. Evol. **10:** 512-526.

Templeton AR, RJ Robertson, J Brisson, J Strasburg. 2001. Disrupting evolutionary processes: the effect of habitat fragmentation on collared lizards in the Missouri Ozarks. Proc. Natl. Acad. Sci. USA **98:** 5426-5432.

Teng LS. 1990. Geotectonic evolution of Late Cenozoic arc-continent collision in Taiwan. Tectonophysics **183:** 57-76.

Thompson J, D Higgins, T Gibson. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting position-specific gap penalties and weight matrix choice. Nucleic Acids Res. **22:** 4673-4690.

Tzeng CS. 1986. Distribution of the freshwater fishes of Taiwan. J. Taiwan. Mus. **39:** 127-146.

Tzeng CS, CH Yang. 2003. Population monitoring and ecological investigation of Formosan landlocked salmon *Oncorhynchus masou formosanus* (VI). Miaoli, Taiwan: Shei-Pa National Park. (in Chinese with English abstract)

Uphyrkina O, D Miquelle, H Quigley, C Driscoll, SJ O'Brien. 2002. Conservation genetics of the Far Eastern leopard (*Panthera pardus orientalis*). J. Hered. **93:** 303-311.

Wang HY, MP Tsai, MJ Yu, SC Lee. 1999. Influence of glaciation on divergence patterns of the endemic minnow, *Zacco pachycaphalus*, in Taiwan. Mol. Ecol. **8:** 1879-1888.

Wang JP, KC Hsu, TY Chiang. 2000. Mitochondrial DNA phylogeography of *Acrossocheilus paradoxus* (Cyprinidae) in Taiwan. Mol. Ecol. **9:** 1483-1494.

Watanabe M, YL Lin. 1985. Revision of the salmonid fish in Taiwan. Bull. Biogeogr. Soc. Jpn. **40:** 75-84.

Watterson GA. 1975. On the number of segregation sites in genetical models without recombination. Theor. Popul. Biol. **7:** 256-276.

Yang Z 1994. Maximum likelihood phylogenetic estimation from DNA sequences with variable rates over sites: approximate methods. J. Mol. Evol. **39:** 306-314.

Yasue Y. 1982. The specificity of Formosan landlocked salmon of Tachia River in Taiwan. *In* E Kimura, ed. Special on freshwater fishes; *Oncorhynchus masou* et *O. rhodurus*. Japan: Society of Freshwater Fishes Protection, pp. 143-147. (in Japanese)

Zawko G, SL Krauss, KW Dixon, K Sivasithamparam. 2001. Conservation genetics of the rare and endangered *Leucopogon obtectus* (Ericaceae). Mol. Ecol. **10:** 2389-2396.