

Phylogeography of the Endangered Species, *Sinogastromyzon puliensis* (Cypriniformes: Balitoridae), in Southwestern Taiwan Based on mtDNA

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Phylogeography of the endangered species, *Sinogastromyzon puliensis* (Cypriniformes: Balitoridae), in southwestern Taiwan based on mtDNA. *Zoological Studies* 47(4): 383-392. The initial colonization by *Sinogastromyzon puliensis* is hypothesized to be in southern Taiwan, followed by its northward dispersal. To unravel its phylogeographical history, the mitochondrial control regions of *S. puliensis* were sequenced. Hierarchical analysis of molecular variance (AMOVA) revealed that more genetic variation resided among populations ($\Phi_{ST} = 0.879, p = 0$) than within populations ($\Phi_{SC} = 0.871, p = 0$), as supported by significant pairwise F_{ST} values showing high genetic differentiation and rare migrations among populations. High levels of genetic differentiation can be explained by either long-term isolation among populations or a consequence due to the founder effect. Both a Neighbor-joining (NJ) tree and maximum parsimony (MP) analysis divided 4 populations of *S. puliensis* into 3 major groups. Group I is composed of populations of the Wu and Choshui Rivers, in which reciprocal monophyly was not supported. The Tzengwen River, comprising group II, is sister to group I. Group III is the basal clade and consisted of only the Kaoping River in a phylogram rooted by *S. wui*. According to the topological pattern and character of haplotypes without a 16-bp deletion, the Kaoping River is proposed to be the first habitat colonized by the ancestor of *S. puliensis*, after which this fish dispersed northwards. This dispersal pattern is concordant with the previously proposed hypothesis.
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Key words: Zoogeography, Dispersal, Glaciation, Founder effect, mtDNA D-loop.

Phylogeography has had significant influence upon historical ecology and population genetics by making it possible to assess the effects of historical events on the genetic composition and structure of modern populations (Bernatchez and Wilson 1998). The most significant contributions were suggested to provide historical dimensions for evolutionary, ecological, and applied studies (Bernatchez and Wilson 1998). Among vertebrates, many studies have focused on fish, largely due to requirements of fisheries management and the island-like habitats of freshwater fish. This latter

feature provides great opportunities for making comparisons among different habitats (Ward et al. 1994, Bernatchez and Wilson 1998, Aurelle et al. 2002).

East Asia is a vast area with a great diversity of freshwater fishes. The phylogeography of freshwater fishes in this area has always been an interesting issue for biologists. However, except for extensive systematic studies in East Asia, the phylogeographic knowledge of this area is rather limited. Compared with this vast area, the phylogeographic history of Taiwan is relatively

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well known. Distribution patterns of several freshwater species in Taiwan were employed to interpret dispersal trends in early studies (Oshima 1923, Tzeng 1986). Recently, phylogeographic histories of some species have been inferred from molecular data (Wang et al. 1999, Wang et al. 2000 2004, Wang 2004, Wang et al. 2007a b). Oshima (1923) proposed a hypothesis based on ichthyofaunal similarities among rivers of Taiwan and adjacent areas, in which freshwater fishes first immigrated into northern or southern Taiwan during glaciations, and then respectively dispersed southward or northward. Oshima (1923) divided freshwater fishes into 2 major groups. Those that immigrated via southern Taiwan are of the Indian Region, and the others which arrived via northern Taiwan originated from the Europe-Asiatic Region. Tzeng (1986) followed Oshima's perspective on the 2 origins and expanded the hypothesis with species of Taiwan described after the middle of the 20th century. Furthermore, Tzeng (1986) established 10 distribution patterns and 3 zoogeographic districts based on freshwater

ichthyofaunal similarities in Taiwan. In contrast to the northern and southern origins, a 3rd route was proposed according to molecular data. *Acrossocheilus paradoxus* (Günther 1868) is documented as having originated in west-central Taiwan based on the divergence of mitochondrial (mt) DNA among populations (Wang et al. 2000).

Species of *Sinogastromyzon* are widely distributed from Laos to southern China and can be found in the Red River (*Honghe*, China and Vietnam), upper Yangtze River (*Changjiang*, China), Pearl River (*Zhujiang*, China), and several rivers of southwestern Taiwan. Eight of 10 species are found in southern China and Taiwan (Chen 1978, Kottelat 2001a b). The distribution range of this genus is disjunct, forming a gap between Taiwan and the Pearl River of southern China (Fig. 1). Species of *Sinogastromyzon* that inhabit both sides of this gap are *S. puliensis* Liang 1974 in southwestern Taiwan and *S. wui* Fang 1930 in the Pearl River. Neither species is sympatric with congeners. *Sinogastromyzon puliensis* is an endangered endemic species and is distributed in

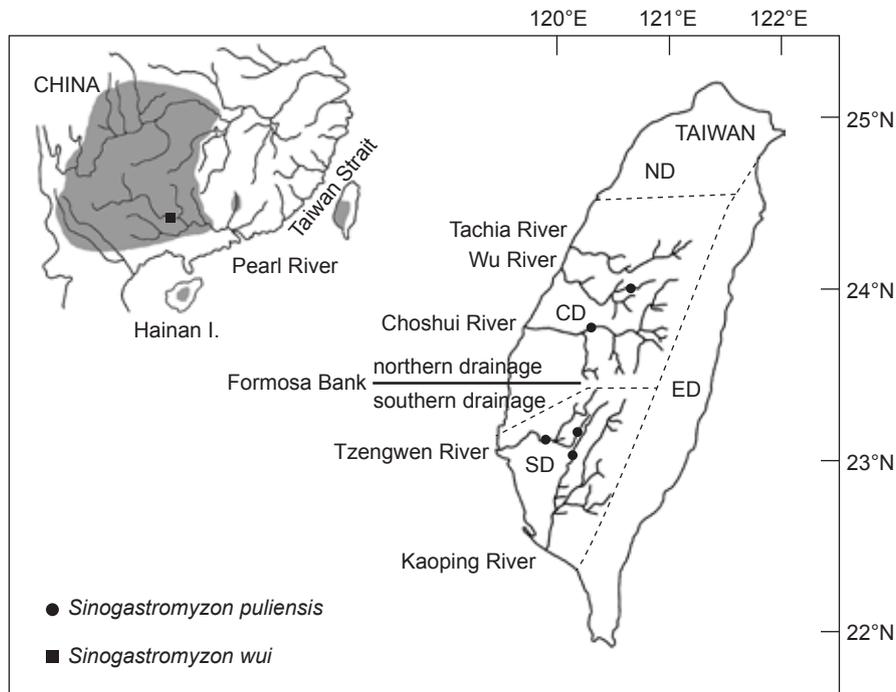


Fig. 1. Sampling localities of *Sinogastromyzon puliensis* and *S. wui*. Zoogeographic districts were those defined by Tzeng (1986): northern district (ND), central district (CD), southern district (SD), and eastern district (ED). The dotted line indicates the border of each district. Shaded areas refer to the approximate distribution range of the genus *Sinogastromyzon*. The Formosa Bank divided the land of the Taiwan Strait, which emerged during the glaciations, into northern and southern drainages. Rivers to the north of the Formosa Bank flowed northward into the Pacific Ocean, while those to the south of the Formosa Bank ran southward into the South China Sea during the glaciations.

5 rivers of southwestern Taiwan (Watanabe 1983, Tzeng 1986, Shen 1993, Lee 2000, Liao et al. 2003). This species is under threat due to habitat destruction and, hence, has been protected by the *Wildlife Conservation Act* of Taiwan since 1989 (Su 2004). Tzeng (1986) proposed a dispersal trend for *S. puliensis* according to the distribution pattern of this genus and conjectured that *S. wui* is its sister species. Tzeng hypothesized a model of a southern origin, in which the southern part of Taiwan was the first area where the ancestors of *S. puliensis* colonized, after which the species dispersed northwards.

To test this hypothesis of a southern origin, our study relied on molecular technologies that have been widely utilized to investigate phylogeographic issues in past years (Hillis et al. 1996, Li 1997). The mitochondrial displacement loop (D-loop) is the most variable marker of the mitochondrial genome. It is highly sensitive to population differentiation, rendering this gene suitable for addressing phylogenetic questions at the population level (Brown et al. 1986, Lee et al. 1995, Faber and Stepien 1997, Sturmbauer et al. 1997). Our study used complete mt D-loop sequences to reconstruct the phylogeography of *S. puliensis*. The aims of our study were to test the southern origin hypothesis of *S. puliensis* proposed by Tzeng (1986) and to provide insights into the formation of the modern ichthyofauna of southern Taiwan.

MATERIALS AND METHODS

Sampling

The number of *S. puliensis* allowed to be collected for research is highly restricted by the Council of Agriculture, Taiwan due to its endangered status. *Sinogastromyzon puliensis* has been recorded in the Tachia, Wu, Choshui, Tzengwen, and Kaoping Rivers of west-central and southwestern Taiwan (Watanabe 1983, Tzeng 1986, Shen 1993, Lee 2000, Liao et al. 2003). Forty-seven samples of *S. puliensis* were collected from the Beigang (a branch of Wu River), Choshui, Tzengwen, Houjue (a major branch of the Tzengwen River), and Nanzixian Rivers (a major branch of the Kaoping River). No *S. puliensis* was found in the Tachia River, although 2 individuals were found in a branch of the Tachia River in 1997 (Lee 2000) where the habitat has suffered from total destruction due to massive construction of riverside embankments. As an outgroup for the phylogenetic analysis, 4 samples of *S. wui* were collected from the Hongshui River, an upstream reach of the Pearl River, southern China (Fig. 1, Table 1). All fish were brought back to the laboratory and preserved in 75% ethanol.

DNA extraction, PCR, and sequencing

A piece of pectoral fin or pelvic fin, weighing about 50 mg, was immersed in 500 μ l digestion buffer (10 mM Tris-HCl (pH 8), 1% SDS, 2 mM

Table 1. Sampling locations, sample size, haplotype diversity (h), and nucleotide diversity (d_{ij}) of *Sinogastromyzon puliensis* in Taiwan. Letters in parentheses indicate abbreviations of zoogeographic districts and streams. Numbers in the parentheses of sample size refer to the number of haplotypes. One common haplotype was shared by W and CS so that the total number in the parentheses of each river is 31 rather than 30

	Sample size	$h \pm SE$	$d_{ij} \pm S.E. (\%)$
Wu (W)	6 (6)	1.0 \pm 0.10	0.59 \pm 0.10
Choshui (CS)	13 (9)	0.86 \pm 0.09	0.36 \pm 0.08
Central region (C; W+CS)	19	0.91 \pm 0.06	0.45 \pm 0.07
Tzengwen (TW)	16 (5)	0.49 \pm 0.12	0.07 \pm 0.03
Kaoping (KP)	12 (11)	0.99 \pm 0.04	0.84 \pm 0.10
Southern region (S; TW+KP)	28	0.84 \pm 0.06	1.95 \pm 0.18
Total	47 (30)	0.93 \pm 0.03	2.0 \pm 0.11

EDTA, 10 mM NaCl, 10 mg/ml DTT, and 0.5 mg/ml proteinase K; modified from Kocher et al. 1989). Preparations were incubated for 16 h at 50°C in a dry bath. DNA was isolated and purified by a phenol/chloroform-isoamyl alcohol extraction procedure (Innis et al. 1989). Six primers were designed for amplification, proofreading, and sequencing of the D-loop: 5'-GTCGACTCTCACCC CTGGCTCCCAAAG-3' for L16530, 5'-ATCTCAAG TGCATAATACATCCT-3' for L16281, 5'-AGTAAGA AACCACCAACCAG-3' for L253, 5'-GGGCATTCT CCACGGGGATGCG-3' for H1026, 5'-AAAGGAAA ACCAAAAAGAGATACG-3' for H519, and 5'-CATG TGTAAGTTGTGCTAAAG-3' for H985. The letters, L and H, respectively refer to light and heavy strains; numbers are associated with the position of the 3' end of the primer on the complete mtDNA of *Formosania lacustre* (Steindachner 1908) (accession no.: NC 001727, Tzeng et al. 1992). A polymerase chain reaction (PCR) was carried out in a thermal cycler (Hybaid OmniGene, Franklin, MA, USA) with 35 cycles of denaturing at 93°C for 30 s, annealing at 50°C for 30 s, and an extension at 72°C for 40 s, followed by a final extension at 72°C for 10 min, after which the samples were maintained at 4°C. PCR products were eluted by a Gene-Spin Gel Extraction Kit (Protech Technology, Taipei, Taiwan) and used for cycle-sequencing with BigDye (Promega, Madison, WI, USA) under the manufacturer's recommended conditions with minor modifications. The sequencing reaction products were screened at the final stage in an automatic sequencer (ABI 377, PE BioSystems, Foster City, CA, USA).

Phylogenetic and population analyses

Sequence alignment was carried out using DNASTar (DNASTAR, Madison, WI, USA) and manually double-checked. Pairwise estimates of genetic distances, based on substitutions among samples, were calculated by the Tamura-Nei method (Tamura and Nei 1993), and a Neighbor-joining (NJ) tree (Saitou and Nei 1987) was evaluated with 1000 bootstraps using unweighted characters (Felsenstein 1985) in the MEGA software (Molecular Evolutionary Genetic Analysis, vers. 3.1, Kumar et al. 2004). In addition, maximum-parsimony (MP) analyses with 1000 bootstraps were conducted by PAUP vers. 4.0b10 (Swofford 2002) using a random addition heuristic search with the tree-bisection-reconnection (TBR) procedure. The gap mode uses pairwise deletions, and the number of

repetitions of random additions is 100. A genetic distance dataset was also employed to construct a minimum spanning network by MINSPNET (Excoffier and Smouse 1994), which provides inferences about the connections of haplotypes among populations of *S. puliensis*. Therein, sequences were grouped into higher clades after linking the affined haplotypes, and then closely related clades were further linked to each other to form a network (Chiang and Schaal 1999, Wang et al. 2000). The minimum spanning network, in which the interior clade represents the ancestor group (Crandall and Templeton 1993), was originally designed for research at the intraspecific level due to the absence of interspecific gene flow (Excoffier and Smouse 1994). In our study, however, only 4 populations were available for network construction, and it would have been relatively more ambiguous to have determined which one was interior. Furthermore, fish species are generally described based on the phylogenetic species concept (Kullander 1999), which means that interspecific gene flow between sister species remains possible. Hence, an outgroup was also included in the network.

A nested clade analysis was used to infer the interrelationships among contemporary and historical processes potentially responsible for the observed mtDNA variations. Geographical associations among clades were assessed using Geodis vers. 2.0 (Posada et al. 2000), and the biological causes for the haplotype-geography associations were inferred from the rules of Templeton (2004).

Haplotype (h , Nei and Tajima 1983) and nucleotide diversities (d_{ij} , Jukes and Cantor 1969) were used to quantify the genetic diversity within populations, zoogeographic districts, and species by DnaSP in a hierarchical manner (vers. 3.0, Rozas and Rozas 1999). DnaSP was also used to estimate genetic variability among populations and between zoogeographic districts (F_{ST} and Nm , Hudson et al. 1992).

Hierarchical partitioning of the genetic diversity was evaluated by analysis of molecular variance (AMOVA) (Excoffier et al. 1992) with the aid of Arlequin 3.000 (Schneider et al. 2000). In the hierarchical analysis, populations were grouped according to zoogeographic districts. In addition, Mantel tests were used to test the correlation between genetic and geographic distances among populations (Manly 1986).

A likelihood-based molecular dating was carried out using the r8s program (Sanderson

2002). PHYLIP vers. 3.6a2 (Felsenstein 2001) was implemented to estimate branch lengths based on the maximum-likelihood criterion with a general time-reversible model. The best tree found by PHYLIP was applied to r8s to execute an unconstrained penalized likelihood analysis with the Powell algorithm (Sanderson 2002).

RESULTS

Divergence and diversity of *Sinogastromyzon puliensis*

In total, 51 mitochondrial D-loop sequences with lengths ranging from 885 to 902 bp were obtained. Four of them were from *S. wui*, and the rest were from *S. puliensis*. Variations in the lengths were primarily due to a deletion of 16 base pairs, located at the left domain of sequences

from *S. puliensis* of the Wu River (W; central zoogeographic district), Choshui River (CS; central zoogeographic district), and Tzengwen River (TW; southern zoogeographic district). All sequences were submitted to GenBank with accession nos. AY344542-AY344573 for *S. puliensis* and AY344574-AY344577 for *S. wui*. The composition of the nucleotide sequences of *S. puliensis* was A-T rich (66.6%) as observed in those of other balitorids (Tzeng et al. 1992, Perdices and Doadrio 2001, Taylor and Verheyen 2001). In total, 30 haplotypes were identified from 47 *S. puliensis* specimens. None of them was shared among populations except haplotype CS3, which occurred in both the Wu and Choshui Rivers, and was more abundant in the latter (1: 4). Haplotype diversities were > 0.49% at all levels (Table 1).

Nucleotide diversities within populations ranged from 0.07% (TW) to 0.84% (the Kaoping River, KP), with an average of 0.47%. The intra-

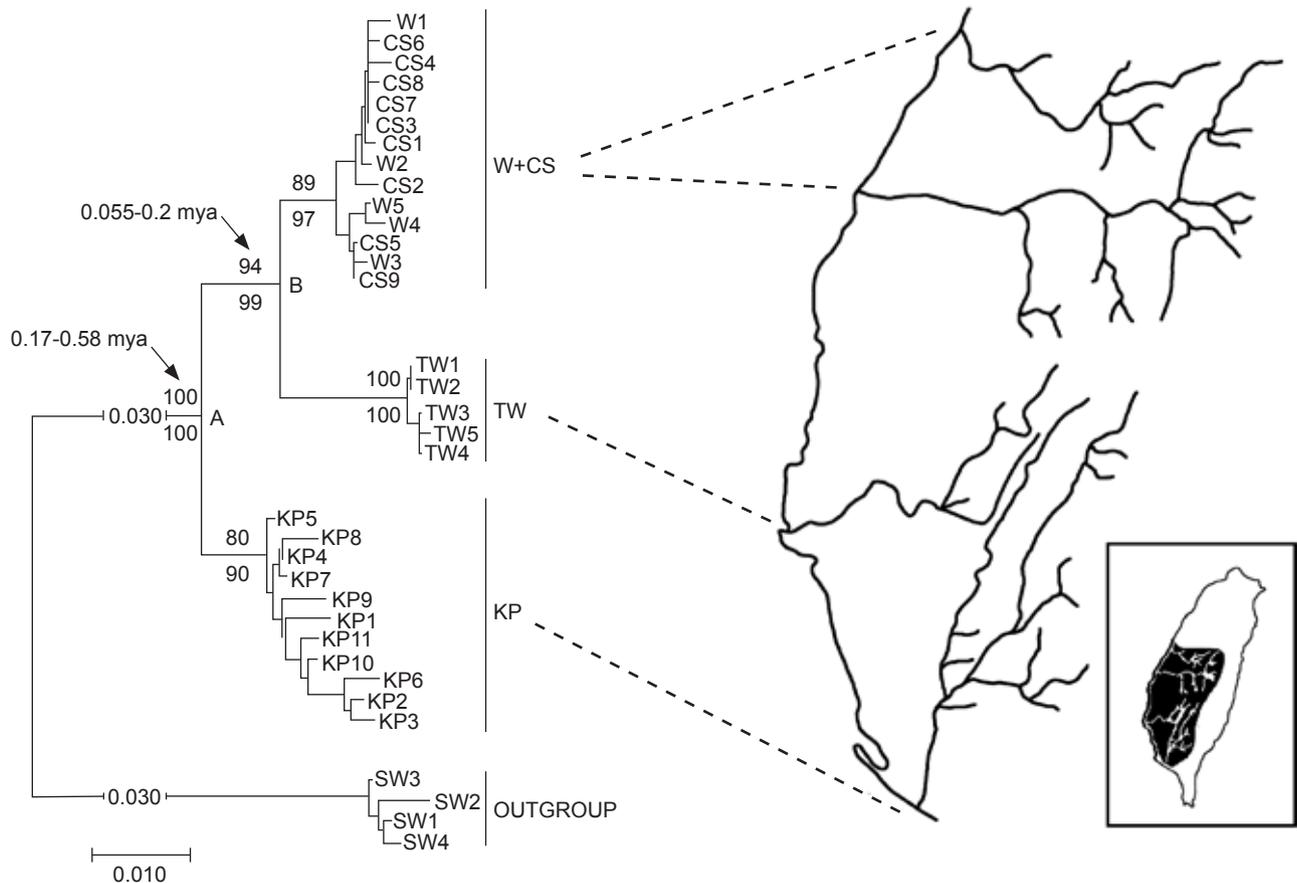


Fig. 2. Neighbor-joining tree and maximum-parsimony tree based on displacement (D)-loop sequences of *Sinogastromyzon puliensis* with *S. wui* as the outgroup. For abbreviations of streams refer to table 1. Bootstrap values of the major branches are shown. Lower and upper values respectively refer to the NJ and MP trees.

district nucleotide diversity of the central region (0.45%) was much lower than that of the southern region (1.95%) and that of all localities (2.0%; Table 1). Genetic distances ranged 0.11%-4.01% among sequences. At the inter-population level, the genetic distance between the Tzengwen (southern zoogeographic district) and Kaoping Rivers (southern zoogeographic district) was the highest (3.73%), and that between the Wu and Choshui Rivers of the central zoogeographic district was the lowest (0.51%). The genetic distance between the central and southern districts was 2.81% and the average among all populations was 2.35% (Table 2).

Analyses of geographic division strongly indicated high genetic differentiation among populations. F_{ST} values derived from any 2 populations, except for that between the Wu and Choshui Rivers, were > 0.766 . On the other hand, the low N_m values implied that migrations rarely occurred among populations, aside from the one between the Wu and Choshui Rivers (Table 2). The AMOVA revealed that most of the variation was between populations within groups (81.15%), followed by variation within species (12.00%), and the remaining variation was between groups (6.85%; Table 3). The Mantel test (Manly 1986) showed no association of genetic with geographic divergence ($r = 0.0033$, $p = 0.31$).

Phylogeographic patterns

The NJ tree rooted by *S. wui* exhibited monophyly of *S. puliensis* which was highly supported by a bootstrap value of 100%, and all bootstrap values for the major branches were $> 90\%$. The NJ tree shows the 4 populations of *S. puliensis* to be divided into 3 major groups (Fig. 2). Group I was composed of populations of the Wu and Choshui Rivers, in which reciprocal monophyly was not supported; the Tzengwen

Table 2. Pairwise genetic variability among populations (F_{ST}), genetic distance (above the diagonal), and genetic variability between zoographic districts (N_m) (below the diagonal) of *Sinogastromyzon puliensis*. Numbers before the dashes denote F_{ST} , while the others denote genetic distances (%)

	W	CS	TW	KP
W		0.045/0.51	0.859/2.54	0.766/3.28
CS	10.62		0.906/2.49	0.799/3.21
TW	0.08	0.05		0.861/3.73
KP	0.15	0.13	0.07	

W, Wu River; CS, Choshui River, TW, Tzengwen River; KP, Kaoping River.

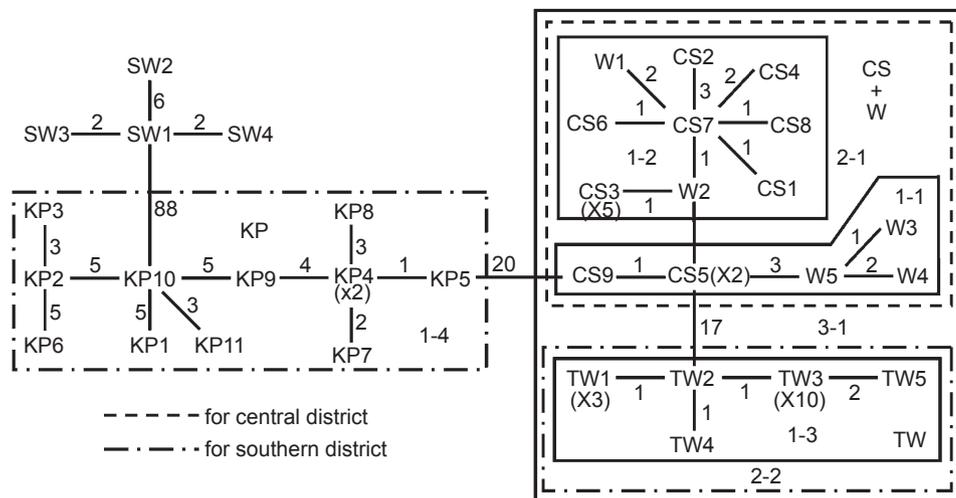


Fig. 3. Minimum spanning network generated by MINSNET using the Excoffier and Smouse method (1994) based on haplotypes of displacement (D)-loop sequences of *Sinogastromyzon puliensis*. Abbreviations are defined in table 1. Numbers between the nodes denote nucleotide substitutions between haplotypes (not to scale). Replicate numbers are attached to the codes of haplotypes when there is more than 1.

River, comprising group II, was sister to group I; group III was the basal clade and consisted of only the Kaoping River in the phylogram rooted by *S. wui*. The phylogram of the MP tree was similar to NJ tree at all levels.

The minimum spanning network (Fig. 3) was largely consistent with both the NJ and MP trees. The outgroup, *S. wui*, was directly connected to haplotypes of the Kaoping River (KP). The 4 populations of *S. puliensis* were further divided into 3 clades. The Wu and Choshui Rivers belonged to the same clade (clade 2-1). CS3 was the only haplotype shared by these 2 populations of the central zoogeographic district, among which 4 replicates occurred in the Choshui River and 1 in the Wu River. Haplotypes of the Tzengwen and Kaoping Rivers formed the other 2 monophyletic clades, clades 1-3 and 1-4, respectively. Notably,

direct linkage between haplotypes of the Tzengwen and Kaoping Rivers of the southern zoogeographic district was absent. They were obliquely connected via the clade composed of the Wu and Choshui Rivers.

The statistical results generated by Geodis (Table 4) referred to the latest inference key of Templeton (2004). Results of the nested clade analysis were interpreted as past fragmentation and/or long-distance colonization for clade 3-1.

For estimates of separation times, the divergence rate was set to 3.2%-10.4%/million yr (ma) as inferred from *Varicorhinus barbatulus* (Pellegrin 1908) (Wang et al. 2004). The separation time calculated by r8s among populations of *S. puliensis* was approximately 0.055-0.2 million year ago (mya) and 0.17-0.58 mya for nodes A and B, respectively (Fig. 2).

Table 3. AMOVA results for testing genetic subdivision between populations of *Sinogastromyzon puliensis* in Taiwan using Arlequin 2000

Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation	Fixation indices
Among groups	1	201.995	1.19507 Va	6.85	Φ_{SC} : 0.87114
Among populations within groups	2	314.742	14.16438 Vb	81.15	Φ_{ST} : 0.87996
Within populations	43	90.093	2.09518 Vc	12.00	Φ_{CT} : 0.06847
Total	46	606.830	17.45463		

Table 4. (a) Nested clade distance analysis of mitochondrial (mt) DNA haplotypes observed in *Sinogastromyzon puliensis*. D_C and D_N are clade and nested clade distances, respectively. The superscripts, S and L, indicate that distance measures are significantly smaller and larger, respectively, than expected values under a random distribution of haplotypes. (b) Demographic inferences from the nested clade distance analysis of *S. puliensis*

(a)

2-step	D_C	D_N	3-step	D_C	D_N
2-1 _I	0.000 ^S	51.04 ^S	3-1 _T	57.19	58.44 ^L
1-3 _T	334.9 ^S	67.87 ^L	1-4 _T	0.000 ^S	51.49
I/T	21.927 ^L	-16.82 ^S			

(b)

Clade	Permutational χ^2	P	Clade key	Inferences
2-step clades nested in 3-1	30.00	0.000	1-2-3-5-15-NO	Past fragmentation and/or long-distance colonization

DISCUSSION

Genetic divergence among populations

Genetic distances derived from the D-loop by the Tamura-Nei method exhibited a trend associated with high divergence among populations (with a mean of 2.63%) and low diversity (with a mean of 0.47%) within populations, which are similar to values for other freshwater fishes of Taiwan (Lin and Huang 1999, Wang et al. 1999, Wang et al. 2000). In addition, hierarchical AMOVA also revealed a similar trend, in which greater genetic variation resided among populations ($\Phi_{ST} = 0.879$, $p = 0$) than within populations ($\Phi_{SC} = 0.871$, $p = 0$; Table 3), as supported by significant pairwise F_{ST} (Table 2). This might have been due to long-term isolation of primarily freshwater fishes created by marine conditions and/or a consequence of the founder effect (Wang et al. 1999). Since marine conditions are a major influence constraining communication among populations of freshwater fishes, the long-term isolation hypothesis may more likely be the reason for the high divergence among populations.

Our study revealed that the genetic distance of *S. puliensis* decreases with increasing geographic distance (Table 2). The Mantel test (Manly 1986) shows irrelevance between genetic and geographic divergences ($r = 0.0033$, $p = 0.31$). This pattern is not in accordance with the model of isolation by distance (Slatkin 1993), in which the genetic distance increases with geographic distance.

Phylogeography of *Sinogastromyzon puliensis*

Ancestral alleles tend to locate at the basal node of a topology, and populations with ancestral genotypes tend to preserve higher nucleotide and haplotype diversities because of long-term accumulation of mutations (Crandall and Templeton 1993, Chiang and Schaal 1999, Wang et al. 2000). According to the phylogenetic trees, the Kaoping River clade was on the basal node of *S. puliensis* rooted by *S. wui* (Fig. 2). In addition, the 16-bp deletion found only in *S. puliensis* of the Wu, Choshui, and Tzengwen Rivers was absent from haplotypes of the Kaoping River as well as from other closely related balitorines (Wang 2004). Thus, a haplotype without the 16-bp deletion was identified as an ancestral one. Furthermore, the greater nucleotide and haplotype diversities of the Kaoping River also indicate the same conclusion

(Table 1). This molecular evidence supports the Kaoping River possibly being the first river into which the ancestor of *S. puliensis* immigrated, which is concordant with the hypothesis proposed by Tzeng (1986), a scenario different from that for *A. paradoxus* (Wang et al. 2000).

The minimum spanning network, including the outgroup (Fig. 3), shows a direct linkage between the outgroup and the Kaoping River population. This connection implies that the Kaoping River clade is ancestral, which is consistent with the topologies of NJ and MP trees. The minimum spanning network underlies the conjecture of the dispersal routes of *S. puliensis* in southwestern Taiwan. According to the inference of the 1st colonization, the 2nd place where *S. puliensis* colonized would be situated next to the Kaoping River in this network. Therefore, the clade composed of the Wu and Choshui Rivers was probably the 2nd colony. The unique 16-bp deletion, found only in the Wu, Choshui, and Tzengwen Rivers, implies that the dispersal event from the Kaoping to the Choshui and Wu Rivers was probably a result of the founder effect (Boileau et al. 1992). According to the minimum spanning network, immigrants into the Tzengwen River were from the Choshui and Wu Rivers rather than from the Kaoping River (Fig. 3). This phenomenon implies that either the Tzengwen River was skipped when *S. puliensis* migrated northward or there was an extinction event followed by back colonization. This unique dispersal scenario may explain why isolation by distance was not observed in *S. puliensis*. The genetic structure of *A. paradoxus* also reveals that haplotypes of the Tzengwen River originated from rivers of west-central Taiwan (Wang et al. 2000).

The Formosa Bank (which emerged 0.15 mya, Lin 1966) was a land bridge located in the Taiwan Strait in an area between the Choshui and Tzengwen Rivers (Fig. 1). This tectonic barrier may have influenced animal migrations among areas of southwestern Taiwan during glaciations, thus helping shape the genetic structure shown in our study. Likelihood-based molecular dating estimated the separation time of node A to be 0.17-0.58 mya, implying that the divergence of this node was not affected by the presence of the Formosa Bank. On the other hand, the separation time for node B was 0.055-0.2 mya, which implies that the separation of node B probably had begun contemporaneously or later than the emergence of the tectonic barrier. The dating reveals that the Formosa Bank may have played an important

role in the divergence of the Tzengwen River and remaining 2 populations. This conjecture is consistent with the phylogeographical event inferred from the nested clade analysis in which the divergence of clade 3-1 (equal to node B) was due to past fragmentation and/or long-distance colonization.

Our study reveals the phylogeographic history of *S. puliensis*, in which the Kaoping River is inferred to be the first habitat the ancestors of this species colonized. This dispersal pattern is concordant with the hypothesis proposed by Tzeng (1986). A southern origin of *S. puliensis* implies that the disjunct distribution of *Sinogastromyzon* species in southern China is probably a consequence of extinction events. Further studies focusing on the phylogeography of *S. wui* on the other side of the distribution gap would provide more information about the knowledge of this disjunct distribution pattern.

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