



Journal of Fish Biology (2010) **76**, 1173–1189

doi:10.1111/j.1095-8649.2010.02589.x, available online at www.interscience.wiley.com

Multiple factors have shaped the phylogeography of Chinese spiny loach *Cobitis sinensis* in Taiwan as inferred from mitochondrial DNA variation

T.-Y. CHIANG*, H.-D. LIN*, K.-T. SHAO† AND K.-C. HSU‡‡

*Department of Life Sciences, Cheng Kung University, Tainan 70101, Taiwan and

†Biodiversity Research Center, Academia Sinica, Nankang, Taipei 11529, Taiwan

(Received 24 March 2009, Accepted 13 January 2010)

Mitochondrial DNA cytochrome *b* sequences (1140 bp) in 61 specimens of Chinese spiny loach *Cobitis sinensis* from 12 drainages in Taiwan were identified as two major clades, exhibiting a southern and a northern distribution, north of TzengWen and south of TzengWen (including TzengWen), respectively. The divergence time between these two phylogroups was estimated at 7.34–9.06 million years before present (B.P.), but these two phylogroups were formed *c.* 3.41–4.23 and 2.22–2.75 M B.P., respectively. Moreover, geological events have been recalculated that Taiwan Island emerged above sea level at an estimate of *c.* 4–5 M B.P., and quickly became its present shape at *c.* 2 M B.P. through mountain building. These results suggest that these two major clades of *C. sinensis* in Taiwan might originate from two different continental populations, since the island's initial isolation in the Pliocene. Within southern Taiwan, the initial colonization was hypothesized to be in KaoPing River, followed by its northward dispersal. The high divergence between KaoPing and TzengWen was influenced by glaciations and landforms. Within north Taiwan, the colonization was from the Miaoli Plateau through western Taiwan to north-eastern and northern Taiwan. This dispersal pattern is concordant with the previously proposed hypothesis. Apparently, both geological and phylogeographic evidence suggested that river capture of the upper Takia River by the LanYang River promoted range expansion in freshwater fishes and also indicated that the Central Range within Taiwan did not act as a barrier to the dispersal of *C. sinensis*. © 2010 The Authors

Journal compilation © 2010 The Fisheries Society of the British Isles

Key words: *Cobitis sinensis*; glacial event; phylogeography; river capture; Taiwan.

INTRODUCTION

Phylogeography is the phylogenetic analysis of intraspecific genealogies in relation to geography and ecology (Avice *et al.*, 1987). Phylogeographic analysis has become a standard method for deciphering the genetic structures of contemporary populations by providing insights into biogeographic patterns of genetic variation (Avice, 2000). Freshwater fishes dwelling in island-like habitats provide ideal models for phylogeographic analysis because these habitats form a natural link between the geographical and biotic evolution of a distributional range. The geological history of basin interconnections reflects the underlying biogeography of a freshwater species,

‡‡Author to whom correspondence should be addressed. Tel.: +886 227 899545; fax: +886 227883463; email: joekchsu@gate.sinica.edu.tw; joekchsu@yahoo.com.tw

because dispersals depend on direct connections between river basins (Bermingham & Martin, 1998; Bernatchez & Wilson, 1998; Aurelle *et al.*, 2002). The phylogeographic analysis of mitochondrial (mt) DNA variability can provide insights into a freshwater species' natural history, including contemporary and historical dispersal patterns (Culling *et al.*, 2006; Chen *et al.*, 2007).

Taiwan is a subtropical island that provides an excellent opportunity to compare contemporary phylogeographic patterns with biogeographic hypotheses. Taiwan is located off the south-eastern coast of mainland China and is separated from China by the shallow Taiwan Strait. Taiwan was first isolated from the mainland by rising sea levels four to five million years before present (M B.P.) and reached its present shape at *c.* 2 M B.P. through mountain building (Ho, 1986; Hsu, 1990; Teng, 1990; Liu *et al.*, 2000). Geological evidence indicates that landbridges connected the island to the Asian continent three to four times, initially in the Pliocene and possibly two to three times in the Pleistocene (Gascoyne *et al.*, 1979; Fairbanks, 1989; Yu, 1995; Huang *et al.*, 1995). At present, the island has numerous topographically, climatically and ecologically diverse habitats (Lin, 1990; Su, 1992).

In addition to geological evidence, biological studies indicate a close evolutionary relationship between Taiwanese and Chinese continental species. Based on ichthyofaunal similarities among rivers of Taiwan and adjacent areas, Oshima (1923) hypothesized that many freshwater fishes migrated to northern or southern Taiwan during one of the glacial periods. Some fishes dispersed from the Indian Region *via* southern Taiwan, and others dispersed from Eurasia *via* northern Taiwan. Ota (1991, 1997) also postulated from faunistic analysis that landbridges had connected Taiwan to the Asian continent possibly several times. Both geological and faunal analyses suggested that conspecific populations in Taiwan might consist of multiple evolutionary lineages originating from the Asian continent. Phylogeographic studies also suggest that conspecific populations in Taiwan were founded by different colonization events (Wang *et al.*, 1999; Creer *et al.*, 2001). The KaoPing River in southern Taiwan is possibly the first river to be colonized by ancestral populations, before individuals dispersed northwards to the TzengWen River (Tzeng, 1986; Wang *et al.*, 1999; Liao *et al.*, 2008) and beyond *via* the Miaoli Plateau to western, north-eastern and northern Taiwan (Wang *et al.*, 1999; Wang *et al.*, 2004).

Taiwanese orogeny (mountain building) uplifted the longitudinal Central Range to almost 4000 m, an event that may have played an important role in isolating populations on either side of the mountains in western and eastern Taiwan. Distribution patterns of freshwater fishes (Tzeng, 1986) and plants (Chou *et al.*, 1999) are highly correlated with the topographical isolation by the Central Range, and studies of the freshwater crab populations show that even the lower elevation of the south-western extension of the Central Range prevents gene flow between populations of lowland species (Shih *et al.*, 2004, 2006). In contrast, other phylogeographic studies indicate that the Central Range was not a major barrier to dispersal between populations of freshwater fishes (Wang *et al.*, 2004). Geological evidence (Lin, 1957) provides a mechanism for the dispersal of the aquatic species from western and eastern Taiwan *via* upland stream capture of the upper Takia River by the LanYang River.

Several biogeographic studies emphasize the importances of vicariations, due to the uplift of the Central Range, and of dispersals from continental populations (Yu, 1995; Hikida & Ota, 1997; Chou & Lin, 1997; Ota, 1997; Toda *et al.*, 1997, 1998; Wang *et al.*, 1999, 2000, 2004; Creer *et al.*, 2001). The present study examines

hypotheses of evolutionary processes that have influenced genetic differentiation among populations of a well-suited model fish, Chinese (or Siberian) spiny loach *Cobitis sinensis* Sauvage & Dabry de Thiersant. This species is restricted to rivers and occurs widely throughout Taiwan. The bottom-dwelling habits of this fish, with its tendency to burrow and its poor swimming ability, probably limit dispersal. The lack of economic importance and the minor use of this species as bait by anglers make it unlikely that population structure has been altered by human-mediated translocations. Thus, it is likely that the genetic structuring of populations reflects river evolution and geological processes, as for other taxa (Wang *et al.*, 1999, 2000, 2004).

This study aimed to examine the phylogeographical pattern, using mitochondrial cytochrome *b* (*cyt b*) gene sequences, of *C. sinensis* populations in Taiwan to address several questions. First was the periodic breach of Taiwan Strait by low sea level an important factor shaping patterns of genetic structure in *C. sinensis*? If landbridges connect the island to the Asian continent four to six times (Gascoyne *et al.*, 1979; Fairbanks, 1989; Yu, 1995; Huang *et al.*, 1995), do distinct lineages exist within Taiwan? If present, did distinct lineages in Taiwan originate from different ancestral populations? Did *C. sinensis* colonize northern Taiwan *via* the Miaoli Plateau and the KaoPing River in southern Taiwan, as previously postulated (Wang *et al.*, 1999, 2004) (Fig. 1)? Is the uplifted Central Range a barrier to migration between populations of *C. sinensis* from west to east or *vice versa*? Did the capture of the

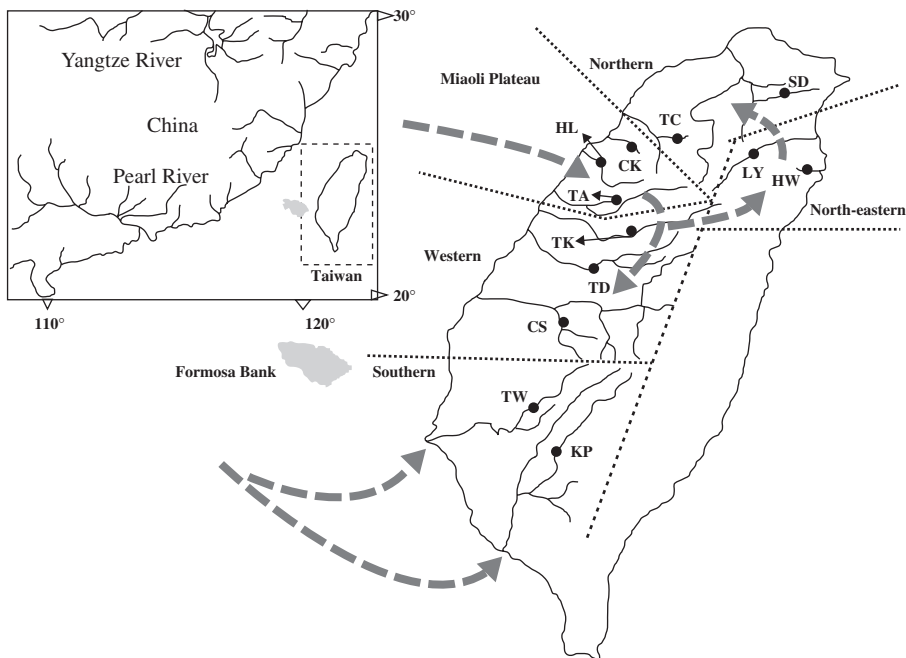


FIG. 1. Sampling localities of *Cobitis sinensis* in Taiwan: CK, ChongKang; CS, ChouShi; HL, HouLong; HW, HsuWao; KP, KaoPing; LY, LanYang; SD, SinDian; TA, TaAn; TC, TouCian; TD, TaDu; TK, TaKia; TW, TzengWen. Zoogeographical districts were those defined by Oshima (1923), Tzeng (1986), Wang *et al.* (1999), Wang *et al.* (2000) and Wang *et al.* (2004). - - -, the border of each district. Possible freshwater fishes migration routes in Taiwan (—), as proposed by Tzeng (1986), Wang *et al.* (1999) and Wang *et al.* (2004).

upper Takia River by the LangYang River promote population expansion from west to east, as supported by geological evidence (Lin, 1957)?

MATERIALS AND METHODS

POPULATION SAMPLING AND MOLECULAR METHODS

A total of 61 specimens of *C. sinensis* from 12 drainages were sampled over most of the species' range in Taiwan (Fig. 1). Locality data and sample sizes appear in Table I. In the ichthyofauna classification of Taiwan (Oshima, 1923; Tzeng, 1986; Wang *et al.*, 1999, 2000, 2004), these 12 drainages belonged to five ichthyofauna subregions: north-eastern subregion (LanYang and HsuWao), northern subregion (SinDian and TouCian), Miaoli Plateau subregion (ChongKang, HouLong and TaAn), western subregion (Takia, TaDu and ChouShi) and southern subregion (TzengWen and KaoPing). Fish were collected with seines and lethally anaesthetized with MS-222 (Sigma; www.sigmaaldrich.com). *Paramisgurnus dabryanus* Dabry de Thiersant is closely related to *Cobitis* (Tang *et al.*, 2006) and, hence, was used as an out-group in this study. The related species of *Cobitis*, *Cobitis lutheri* Rendahl and *Cobitis macrostigma* Dabry de Thiersant, were also used as out-groups.

Samples were stored in 100% ethanol. Genomic DNA was extracted from muscle tissue by the standard protocol of Blin & Stafford (1976). The entire *cyt b* gene was amplified using polymerase chain reactions (PCR) and sequenced using primers L14724 (5'-GACTTGAAAAACCACCGTTG-3') and H15915 (5'-CTCCGATCTCCGGATTACAAGAC-3') (Xiao *et al.*, 2001). Each 100 µl PCR reaction mixture contained 10 ng template DNA, 10 µl 10× reaction buffer, 10 µl dNTP mix (10 mM), 10 pmol of each primer and 4 U of Taq polymerase (Promega; www.promega.com). PCR was programmed on an MJ Thermal Cycler as one cycle of denaturation at 95° C for 4 min, 30 cycles of denaturation at 94° C for 45 s, annealing at 48° C for 1 min 15 s and extension at 72° C for 1 min 30 s, followed by 72° C extension for 10 min and 4° C for storage. PCR products were purified by electrophoresis in a 1.0% agarose gel using 1× tris-acetate-EDTA (TAE) buffer. The gel was stained with ethidium bromide, and the desired DNA band was cut and eluted using the agarose gel purification kit (Qiagen; www.qiagen.com). Products of the cycle-sequencing reactions were run on an ABI 377 automated sequencer (Applied Biosystems; www.appliedbiosystems.com). Chromatograms were checked with CHROMS (Technelysium; www.technelysium.com.au), and sequences were manually edited using BIOEDIT 6.0.7 (Hall, 1999). Sequences were deposited in GenBank under accession numbers AM711121-AM711124, AM910624-AM910633, AM922265-AM922282, AM922487-AM922505 and AM924168-AM924181.

SEQUENCE ALIGNMENT AND PHYLOGENETIC INFERENCES

Nucleotide sequences were aligned using Clustal X 1.81 (Thompson *et al.*, 1997) and corrected by eye. The substitution model used for the phylogenetic reconstructions was estimated with MODELTEST (version 3.06; Posada & Crandall, 1998) using AIC and Bayesian information criteria (BIC) (Posada & Buckley, 2004). The best model selected was GTR. Haplotype genealogy was generated by a neighbour-joining (NJ) analysis with maximum composite likelihood distance model in MEGA4 (Tamura *et al.*, 2007). Maximum-likelihood (ML) trees were made with Data Analysis in Molecular Biology and Evolution (DAMBE 5.0.37; Xia & Xie, 2001). Branch confidence values were estimated using 1000 bootstrap replications. Bayesian inference (BI) phylogenetic inference was performed using MrBayes 3.0b4 (Huelsenbeck & Ronquist, 2001). One million generations were run under the GTR+G+I model of evolution (sample frequency every 100, chain temperature 0.2). The posterior probability values were used as support for the Bayesian topology. Log-likelihood stability was reached after *c.* 35 000 generations, and the first 350 trees were excluded as burn-in. The remaining trees were used to compute a 50% majority rule consensus tree. The number of mutations between DNA haplotypes in pair-wise comparisons was calculated using MEGA4

TABLE I. Samples of *Cobitis sinensis* used for mtDNA analyses, location, abbreviation (Abbr.) and summary statistics

Population	Abbr.	Localities	Samples size (<i>N</i>)	Number of haplotype	Haplotype diversity (<i>h</i>)	Nucleotide diversity (%)	
						θ_{π}	θ_w
Northern Taiwan							
ChongKang	CK	120° 42' E; 24° 35' N	54	46	0.993	1.998	2.331
HouLong	HL	120° 40' E; 24° 25' N	6	6	1.000	1.269	1.537
TaAn	TA	120° 41' E; 24° 15' N	6	5	0.933	0.205	0.269
TaKia	TK	120° 42' E; 24° 10' N	7	6	0.952	0.615	0.753
TaDu	TD	120° 40' E; 24° 00' N	6	5	0.933	0.310	0.384
ChouShi	CS	120° 37' E; 23° 45' N	6	6	1.000	0.292	0.384
SimDian	SD	121° 18' E; 24° 55' N	4	2	0.500	0.351	0.383
TouCian	TC	120° 50' E; 24° 42' N	4	3	0.833	0.322	0.335
LanYang	LY	121° 15' E; 24° 40' N	4	4	1.000	1.243	1.292
HsuWao	HW	121° 20' E; 24° 39' N	5	3	0.700	0.395	0.421
Southern Taiwan			6	6	1.000	0.246	0.269
TzengWen	TW	120° 36' E; 23° 20' N	7	6	0.952	2.059	2.614
KaoPing	KP	120° 38' E; 23° 10' N	1	1	—	—	—
Total			61	52	0.933	0.784	0.730
					0.994	4.383	5.290

and was used to construct a minimum spanning network with MINSPNET (Excoffier & Smouse, 1994).

POPULATION STRUCTURE AND PHYLOGEOGRAPHICAL HISTORY

Estimates of haplotype diversity (h) (Nei & Tajima, 1983) and nucleotide diversity (θ_π , θ_w) followed Nei (1987). Interpopulation nucleotide divergence between samples was estimated with D_a using DnaSP 4.10.8 (Rozas *et al.*, 2003). The current (θ_π) and historical (θ_w) genetic diversity were determined in DnaSP. Comparing estimates generated by these two indices provides insight into population dynamics over recent evolutionary history (Templeton, 1993). Differences between these statistics indicate population decline (if $\theta_\pi < \theta_w$) or growth (if $\theta_\pi > \theta_w$) (Pearse & Crandall, 2004; Buhay & Crandall, 2005). The hierarchical structure of *cyt b* variation was examined with an analysis of molecular variance (AMOVA, Excoffier *et al.*, 1992) using Arlequin 2.000 (Schneider *et al.*, 2000). This analysis was performed with a K2P distance with the gamma correction ($\alpha = 1.242$) and 20 000 permutations. AMOVA partitions the observed variation among samples into within-population (F_{ST}), within-group (F_{SC}) and among-group (F_{CT}) components.

MDIV (Nielsen & Wakeley, 2001), a Bayesian Markov-chain Monte-Carlo (MCMC) method, was used to estimate the posterior distribution of theta ($\theta = 2N_e\mu$), the number of migrants per generation ($M = N_e m$, where m is migration rate) and divergence time between populations (equations adjusted for mtDNA). MDIV was used to estimate the time to the most recent common ancestor (T_{MRCA}) for all sequences. Five MCMC runs were performed with five million steps each, with a 10% burn-in Bayesian analysis, and were also performed with BEAST 1.3 (Drummond & Rambaut, 2005) to investigate the history of *C. sinensis* in Taiwan. Posterior distributions of parameters were estimated using two independent MCMC analyses of 10 million steps each with a burn-in of 10%. Samples from the two chains, which yield similar results, were combined. Convergence of the chains was checked using TRACER 1.4 (Rambaut & Drummond, 2004), and the effective sample size (ESS) for each parameter exceeded 200. Values of T_{MRCA} using this method were compared with estimates of T_{MRCA} from MDIV.

IM (Hey & Nielsen, 2004) was used to determine whether the observed pattern of genetic variation was a result of historical divergence or limited contemporary migration. IM was used in this instance instead of MDIV because it allowed for asymmetric rates of gene flow and different effective population sizes of the source populations. IM produced similar estimates for each run; parameter estimates fell within the 95% highest posterior densities (HPD) for the parameter estimates from the other runs. The mode and 95% HPD for estimates were used from the run producing the largest ESS (Hey & Nielsen, 2004) for the estimates.

RESULTS

SEQUENCE DIVERSITY

A total of 61 *cyt b* sequences [1140 base pairs (bp)] were obtained from 12 populations of *C. sinensis*. A total of 259 sites were variable, of which 198 were parsimoniously informative. Sequences were A+T (59.0%) rich. Base frequencies were A = 0.274, C = 0.262, G = 0.148 and T = 0.316 overall and A = 0.363, C = 0.279, G = 0.068 and T = 0.289 in third-codon positions. These frequencies were strongly biased against guanine. Nucleotide substitutions were biased towards transitions and with a mean ti:tv ratio of 3:4. Estimates of the current (θ_π) and historical (θ_w) genetic diversity per sites for each sample indicated that only the KP sample from southern Taiwan showed a pattern of growth ($\theta_\pi < \theta_w$; Table I).

EVOLUTIONARY RELATIONSHIPS

In the phylogenetic analyses, the use of *P. dabryanus*, *C. lutheri* or *C. macrostigma* produced the same result. The topologies of the ML, NJ and BI trees were identical, with only small differences in bootstrap values (Fig. 2). Fifty-two *cyt b* haplotypes fell into two major lineages; in the consensus NJ tree, the first group contained two samples from the south Taiwan (southern lineage) and the second group contained ten samples from North Taiwan (northern lineage), both with significant bootstrap support. Two sublineages appeared within the north lineage, also with high bootstrap support. The first sublineage included three samples from the Miaoli Plateau, and the second sublineage (non-plateau sublineage) included samples from western, northern and north-eastern Taiwan. The Miaoli Plateau sublineage appeared in a basal position. The genealogies of these two sublineages consisted of short branches and little structure. Three minor lineages appeared within the non-plateau sublineage with high bootstrap support, except for eastern sublineage (Fig. 2). Samples from north-eastern and northern Taiwan clustered within a single lineage.

The haplotype network consisted of three levels (Fig. 3) and was largely consistent with the topology of the phylogenetic tree (Fig. 2). Two disjointed clades, 2-1 and 2-2, corresponded to northern and southern lineages, respectively. Within clade 2-1, the north-eastern populations [clade 1-3; LY and HW (see Fig. 1 or Table 1 for abbreviations)] were linked to the northern (clade 1-4; SD & TC) and western (clade 1-2; TK, TD and CS) clades. The northern and Miaoli Plateau clades were located at tip positions, and the north-eastern and western clades were located at internal positions.

POPULATION STRUCTURE

Hierarchical analysis of molecular variance (AMOVA) indicated significant genetic structure at several levels. The hierarchical analysis of F_{ST} revealed that samples of *C. sinensis* were divided into northern, north-eastern, Miaoli Plateau, western and southern subregions. The largest amount of genetic variation (84.29%) was distributed among, rather than within groups. The AMOVA indicated that most of the genetic variability could be explained by a single north-south partition (Table II). F_{ST} values were >0.959 between two geographic groups. Large fixation indices were also observed in other models, including three to five groups (Table II; Fig. 2). Most of the genetic variation could be explained at the regional level. Because a deep phylogeographic partition was identified between the southern and northern groups, all subsequent analyses of population history were conducted separately for each group, as well as for the total sample.

To assess genetic isolation, the IM programme was used to calculate the amount and direction of gene flow among populations. Within the north lineage, these results suggested asymmetric gene flow from source populations. Gene flow from western to north-eastern populations ($M = 0.10-0.15$) was higher than that from north-eastern to western populations ($M = 0.05-0.10$). The results also indicated low levels of reciprocal gene flow between northern areas and the Miaoli Plateau. Gene flow between populations on the Miaoli Plateau and western areas as well as between northern and north-eastern areas was higher than other comparisons. Conversely, the magnitude of gene flow between western and north-eastern populations was moderate. The results of the nested clade analysis and IM analysis indicate migratory routes from Miaoli Plateau through the Takia River to western Taiwan, then to

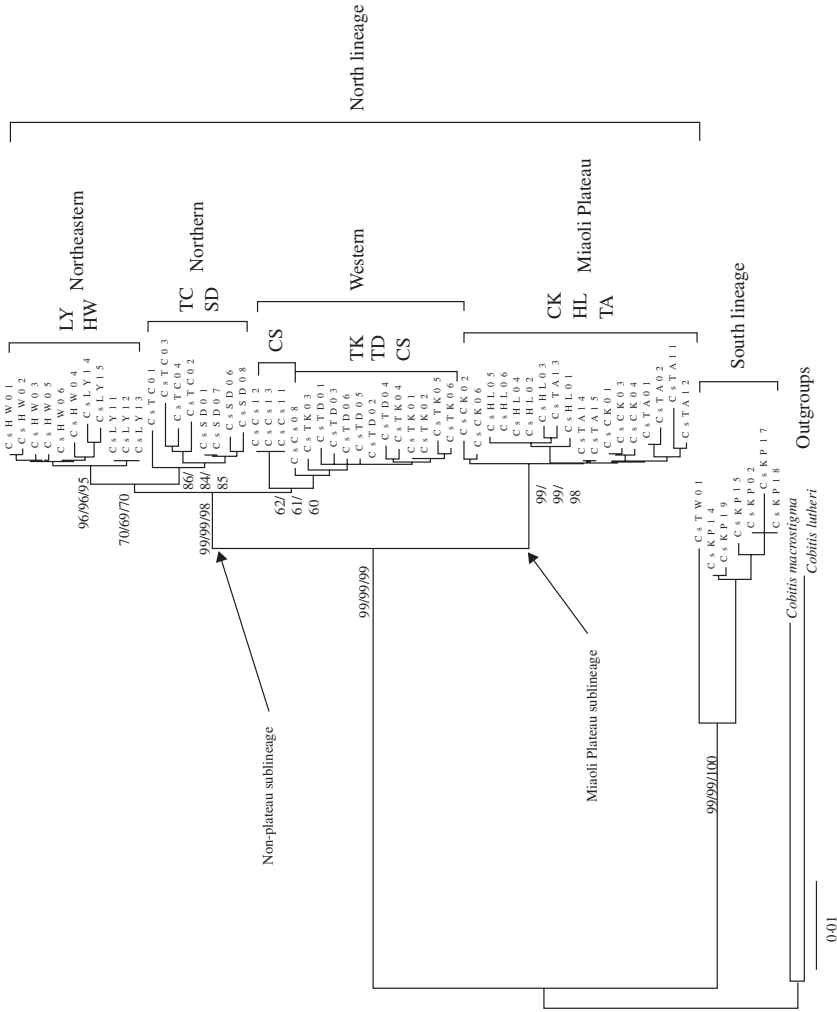


FIG. 2. The *Cobitis sinensis* neighbour-joining (NJ) tree with maximum composite-likelihood distance model based on mitochondrial DNA cytochrome *b* sequences. The numbers at the nodes are bootstrap values of the maximum likelihood (ML), NJ and Bayesian inference (BI) analyses. (See Fig. 1 for sampling site abbreviations.)

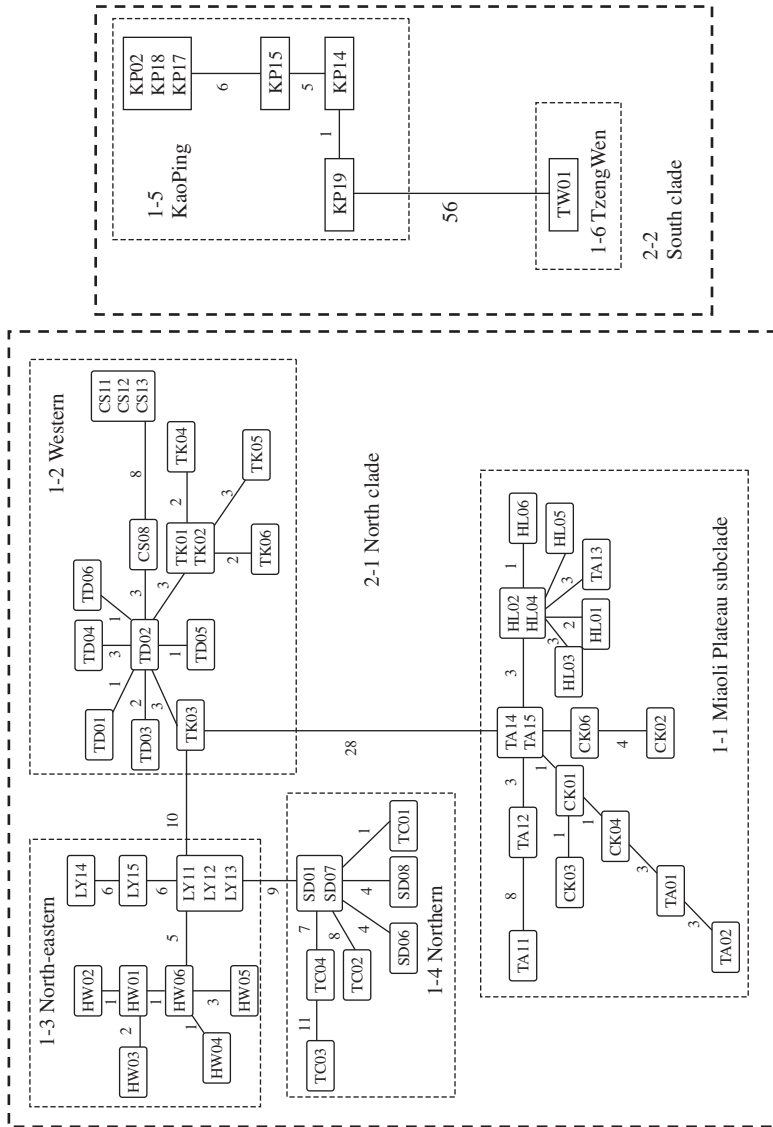


FIG. 3. Minimum spanning network of mitochondrial DNA cytochrome *b* haplotypes in Taiwan *Cobitis sinensis* populations. The two major mtDNA clades (northern and southern) were treated as separate networks. Numbers at nodes indicate number of nucleotide changes between haplotypes. (See Fig. 1 for sampling site abbreviations.)

TABLE II. Analysis of molecular variance (AMOVA)

Clade	Hierarchical groups	% Variance	Statistics	P
Five ichthyological subregions (northern, north-eastern, Miaoli, western and southern)				
	Among regions	84.29	$F_{SC} = 0.516$	<0.001
	Among populations in regions	8.11	$F_{ST} = 0.924$	<0.001
	Within populations	7.60	$F_{CT} = 0.843$	<0.001
Total	Among regions (northern clade v. southern clade)	82.77	$F_{SC} = 0.761$	<0.001
	Among populations in regions	13.11	$F_{ST} = 0.959$	<0.001
	Within populations	4.12	$F_{CT} = 0.828$	<0.001
North	Among regions (Miaoli Plateau v. non-plateau)	64.74	$F_{SC} = 0.536$	<0.001
	Among populations in regions	18.89	$F_{ST} = 0.836$	<0.001
	Within populations	16.38	$F_{CT} = 0.647$	<0.010
Four ichthyological subregions in northern Taiwan (north, north-eastern, western and Miaoli)				
	Among regions	74.47	$F_{SC} = 0.376$	<0.001
	Among populations in regions	9.60	$F_{ST} = 0.841$	<0.001
	Within populations	15.92	$F_{CT} = 0.745$	<0.001
Three ichthyological subregions in non-plateau subclade (north; north-eastern and western)				
	Among regions	48.83	$F_{SC} = 0.406$	<0.001
	Among populations in regions	20.78	$F_{ST} = 0.696$	<0.001
	Within populations	30.39	$F_{CT} = 0.488$	<0.001

Analyses were performed separately for populations in each clade.

the Takia River in north-eastern Taiwan and to north-eastern and northern Taiwan. Within southern Taiwan, the IM analysis indicated no gene flow between KaoPing and TzenWen rivers (Fig. 1).

COALESCENT ANALYSES AND DIVERGENCE TIMES

The mtDNA *cyt b* evolutionary rate could not be estimated for *C. sinensis* because of a lack of a calibration point. Therefore, estimates of T were converted to real time by assuming a range of neutral mutation rates in proxy species. A divergence rate of 0.68–0.84% per million years was previously estimated for *Cobitis* (Perdices & Doadrio, 2001; Doadrio & Perdices, 2005; Culling *et al.*, 2006). Divergence between the northern and southern clades was $T = 2.38$ (MDIV; 95% HPD $T = 0.8–3.6$). Hence, the northern and southern lineages diverged from one another at *c.* 7.330–9.060 M B.P. The estimate of divergence time between the two subclades within the northern clade was $T = 0.17$ (95% HPD $T = 0.1–0.8$) and represented 0.253–0.314 M B.P. The divergence time estimate between the two populations in the southern clade was $T = 2.78$ (95% HPD $T = 1.89–4.36$) and represented 2.050–2.540 M B.P. The Bayesian approach implemented in BEAST yielded an estimate T_{MRCA} for the whole complex at 9.22–11.43 M B.P. (CI: 6.11–13.03 M B.P.), whereas the likelihood approach implemented in MDIV estimated T_{MRCA} at 9.25–11.47 M B.P. The T_{MRCA} of northern and southern clades was estimated at 2.22–2.75 M B.P. and 3.41–4.23 M B.P., respectively.

DISCUSSION

ORIGINATION FROM TWO CONTINENTAL POPULATIONS IN THE PLIOCENE

Taiwan Strait is 70–100 m deep, 130–250 km wide and separates the island of Taiwan from continental China. Previous phylogeographic studies of Taiwanese species have estimated the colonization dates during the landbridge phase in the Pleistocene for freshwater fishes (0.055 Ma for *Zacco pachycephalus* (Günther), see Wang *et al.*, 1999; 0.1–1 M B.P. for *Acrossocheilus paradoxus* (Günther), see Wang *et al.*, 2000) and frogs (0.15–0.24 M B.P. for *Rhacophorus taipeianus* and 0.38–0.45 M B.P. for *Rhacophorus moltrechti*, see Yang *et al.*, 1994). Other phylogeographic studies, however, indicate that Taiwan was colonized by ancestral populations >2 M B.P. (Pliocene; 5.8 M B.P. for the lizard *Takydromus sauteri*, Lin *et al.*, 2002; 5.7 M B.P. for the crab *Candidiopotamon rathbunae*, Shih *et al.*, 2006; 2–3 M B.P. for the snake *Trimeresurus stejnegeri*, Creer *et al.*, 2001). Many species could easily have migrated from the mainland to the island of Taiwan during Pliocene and Pleistocene glaciations as a result of the lowered sea level (Huang *et al.*, 1995). If a Pleistocene colonization by *C. sinensis* is assumed, the substitution rate for the *cyt b* gene would be three-fold larger (3.14% sequence divergence per million years) than values obtained in other studies of *Cobitis* species (0.68–0.84% sequence divergence per million years; Perdices & Doadrio, 2001; Doadrio & Perdices, 2005; Culling *et al.*, 2006). Therefore, the present dataset indicates that *C. sinensis* most probably colonized the island during Pliocene, not Pleistocene, glaciations. An earlier molecular clock calibration indicates that the northern and southern lineages in Taiwan arose *c.* 2.22–2.75 and 3.41–4.23 M B.P., respectively. These estimates imply that the island has been populated by *C. sinensis* for more than four million years, a date that matches the age of the uplift of the island (4–5 M B.P.; Hsu, 1990; Teng, 1990; Liu *et al.*, 2000). The geological history of the area indicates that a landbridge connected the island to the Asian continent initially in the Pliocene (Huang *et al.*, 1995). Phylogeographic and floristic analyses (Shen, 1994; Lin *et al.*, 2002; Shih *et al.*, 2006) also indicate that Taiwan Island has had a biological history of *c.* 5 M B.P. Tao & Hu (2001) also found that Taiwan had Pliocene fossil teeth of several fish species. Therefore, a Pliocene colonization of the island by *C. sinensis* represents one of the earliest records of freshwater fishes on the island.

Lin (1957) proposed that the Formosa Bank, a ridge located in the Taiwan Strait in an area north of TzengWen, influenced animal migrations during glaciations. The Bank divided the glacial landbridge into northern and southern drainages, so that northern rivers flowed northwards into the Pacific Ocean and southern rivers flowed southwards into the South China Sea. Because of the topographical isolation by this bank, Lin (1957) suggested that the Taiwanese fauna was separated into two regions north and south of TzengWen (including TzengWen). The present study found that the distributions of the northern and southern clades of *C. sinensis* in Taiwan are consistent with isolation by the Formosa Bank. The present results agree with those of Oshima (1923), who considered that the freshwater fishes in southern Taiwan originated from the Indian Region and those in northern Taiwan from the Euroasiatic Region. This dispersal and colonization hypothesis agrees with the results of previous studies (Oshima, 1923; Tzeng, 1986; Wang *et al.*, 1999)

and with the current concepts of the tectonic evolution of the island of Taiwan (Lin, 1957).

PHYLOGEOGRAPHY OF *C. SINENSIS*

The mtDNA genealogy and network were mostly consistent with the subregions defined based on their ichthyofauna (Fig. 1; Tzeng, 1986; Wang *et al.*, 2004). The shape of the haplotype network is consistent with a relatively recent population expansion in the north clade, in which several localized lineages are connected by short branches to the most common haplotypes (Fig. 3). In the phylogenetic analysis, the Miaoli Plateau sublineage appeared in a basal position within the northern lineage and implies that these are the oldest populations in the last region isolated from mainland in northern Taiwan. The phylogeography of the cyprinid fish *Varicorhinus barbatulus* (Pellegrin), 1908 (Wang *et al.*, 2004) also indicates that the Miaoli Plateau region was the last region isolated from the mainland. According to coalescence theories, the interior position in the minimum spanning network and high frequencies of the western subregion clade, 1-2, indicates that it was probably an ancestral clade and may represent relict ancestral haplotypes (Crandall & Templeton, 1993). The phylogenetic analyses (Figs 2 and 3) suggest that dispersals occurred from the Miaoli Plateau through the Takia area to central western Taiwan and then from the Takia River to north-eastern (LanYang and HsuWao rivers) and northern (SinDian and TouCian rivers) areas (Fig. 1). Estimates of gene flow also support a migratory route in North Taiwan clade from Miaoli Plateau *via* the Takia River to western, north-eastern and northern Taiwan.

The Central Range, which consists of the Central, Syivia and Yushan mountains, was formed by the collision of the Philippine Sea and continental Asian tectonic plates *c.* 2 M B.P. (Lin, 1966; Page & Suppe, 1981). This mountain range may have acted as a barrier to the dispersal between western and eastern populations in species, such as *C. sinensis*, which have limited migratory capabilities. The mtDNA gene genealogy and the molecular dating from the MDIV analysis, however, indicated that the divergence between the Takia and LangYang populations was very recent. The upper stream regions of the LanYang and Takia Rivers are located on the border between the Central and Syivia mountains; the LanYang River flows north-east into Pacific and Takia River flows west into Taiwan Strait. Topological evidence, however, indicates river capture of the upper Takia River by the LanYang River (Lin, 1957), and this capture probably facilitated the interdrainage transfers, produced vicariant isolations and led to the cladogenesis of freshwater taxa (Burrige *et al.*, 2006; Kozak *et al.*, 2006). The results of the present study indicate that north-eastern and northern populations originated from the Takia River by way of the LanYang River (Fig. 3). Generally, freshwater fishes, *e.g.* *C. sinensis*, *Candidia barbata* (Regan), *Z. pachycephalus*, and *A. paradoxus* (Tzeng, 1986), that inhabit western Taiwan are also found in north-eastern Taiwan. The mtDNA gene genealogy did not support the hypothesis that the Central and Syivia mountains acted as a barrier to the dispersal of freshwater fishes (Wang *et al.*, 1999, 2000, 2004). Therefore, river capture may have played an important role in the biogeography and evolution of freshwater fishes in northern Taiwan.

In the present study, samples from KP and TW formed two high divergence monophyletic clades, clades 1-5 and 1-6, respectively. This is consistent with a

previous genetic study of *Sinogastromyzon puliensis* Liang, (Cypriniformes: Balitoridae) in south-western Taiwan, which showed that the TzengWen population also formed a monophyletic clade, distinct from other populations (Liao *et al.*, 2008). Liao *et al.* (2008) proposed that ancestral populations of *S. puliensis* first colonized the KaoPing River. This hypothesis implies that colonists either skipped the TzengWen River when *S. puliensis* migrated northwards or that a colonization was followed by extinction, and then again followed by back colonization. The landbridge between the mainland and the island may have acted as a refuge during glaciations in the late Pleistocene (Boggs *et al.*, 1979). Rivers south of the KaoPing River, however, did not extend onto the landbridge, because a deep sea trench near the KaoPing estuary reached 200 m depth within 3 km of shore. Additionally, low temperatures would have prevented *C. sinensis* from reaching this area, even though the distance between the upstream reaches of the KaoPing and TzengWen rivers was short. Therefore, the divergent clades in the KaoPing and TzengWen rivers may have arisen under the influences of glaciation and landform. This scenario is also consistent with the results of *C. sinensis* in the present study, which initially colonized KaoPing River then dispersed northwards because of topographic changes during glaciations.

In summary, the sea-level history of Taiwan Strait was important in shaping the genetic structures of populations of *C. sinensis*. The deep mtDNA separations between northern and southern lineages indicate that these lineages had independent origins, since the initial isolation of Taiwan in the Pliocene. The initial colonization in southern Taiwan appears to have been in the KaoPing River, and this was followed by northward dispersal. The large amount of divergence between KaoPing and TzengWen populations is likely to be due to glaciations. Within northern Taiwan, dispersals occurred from the Miaoli Plateau through western Taiwan into north-eastern and northern Taiwan. The NCA and IM analyses indicate that river captures promoted dispersal between eastern and western Taiwan. Uplift of the Central Range has not caused barriers to the dispersal of *C. sinensis* from the west to east and *vice versa*. Climate changes during the Pliocene and Pleistocene Ice Ages and river capture produced barriers and migration pathways that are reflected in the genetic structure of present-day populations. The deep mtDNA divergence between lineages of *C. sinensis* indicates that, for conservation, this species can be divided into two geographical units that can be considered evolutionarily significant units (ESU). The θ_π and θ_w indicate that all populations, excluding the KaoPing population, show decline in population size. The results of this study are important for the development of a management plan for the conservation of freshwater fishes in Taiwan.

We are grateful to W. L. Wu, Y. F. Lee of the Department of Life Sciences, Cheng Kung University, C. S. Tzeng of the Department of Life Sciences, Tsing Hwa University and S. C. Lee of the Institute of Cellular and Organismic Biology, Academia Sinica for some stimulating discussions. Part of this work was carried out using the resources of the Computational Biology Service Unit from Cornell University, which is partially funded by Microsoft Corporation.

References

- Aurelle, D., Cattaneo-Berrebi, G. & Berrebi, P. (2002). Natural and artificial secondary contact in brown trout (*Salmo trutta*, L.) in the French western Pyrenees assessed by allozymes and microsatellites. *Heredity* **89**, 171–183.

- Avise, J. C. (2000). *Phylogeography: The History and Formation of Species*. Cambridge, MA: Harvard University Press.
- Avise, J. C., Arnold, J., Ball, R. M., Bermingham, E., Lamb, T., Neigel, E., Reeb, C. A. & Saunderson, N. C. (1987). Intraspecific phylogeography: the mitochondrial DNA bridge between population genetics and systematics. *Annual Review of Ecology and Systematics* **18**, 489–522.
- Bermingham, E. & Martin, A. P. (1998). Comparative mtDNA phylogeography of neotropical freshwater fishes: testing shared history to infer the evolutionary landscape of lower Central America. *Molecular Ecology* **7**, 499–519.
- Bernatchez, L. & Wilson, C. C. (1998). Comparative phylogeography of nearctic and paleartic fishes. *Molecular Ecology* **7**, 431–452.
- Blin, N. & Stafford, D. W. (1976). A general method for isolation of high molecular weight DNA from eukaryotes. *Nucleic Acids Research* **3**, 2303–2308.
- Boggs, S., Wang, W. C., Lewis, F. S. & Chen, J. C. (1979). Sediment properties and water characteristics of the Taiwan shelf and slope. *Acta Oceanographica Taiwanica* **10**, 10–49.
- Buhay, J. E. & Crandall, K. A. (2005). Subterranean phylogeography of freshwater crayfishes shows extensive gene flow and surprisingly large population sizes. *Molecular Ecology* **14**, 4259–4273.
- Burridge, C. P., Craw, D. & Waters, J. M. (2006). River capture, range expansion, and cladogenesis: the genetic signature of freshwater vicariance. *Evolution* **60**, 1038–1049.
- Chen, X. L., Chiang, T. Y., Lin, H. D., Zheng, H. S., Shao, K. T., Zhang, Q. & Hsu, K. C. (2007). Mitochondrial DNA phylogeography of *Glyptothorax fokiensis* and *Glyptothorax hainanensis* in Asia. *Journal of Fish Biology* **70**, 75–93.
- Chou, W. H. & Lin, J. Y. (1997). Geographical variations of *Rana sauteri* (Anura: Ranidae) in Taiwan. *Zoological Studies* **36**, 201–221.
- Chou, C. H., Huang, S., Chen, S. H., Kuoh, C. S., Chiang, T. Y. & Chiang, Y. C. (1999). Ecology and evolution of *Miscanthus* of Taiwan. *National Science Council Monthly* **27**, 1158–1169.
- Crandall, K. A. & Templeton, A. R. (1993). Empirical tests of some predictions from coalescent theory with applications to intraspecific phylogeny reconstruction. *Genetics* **134**, 959–969.
- Creer, S., Malhotra, A., Thorpe, R. S. & Chou, W. H. (2001). Multiple causation of phylogeographical pattern as revealed by nested clade analysis of the bamboo viper (*Trimeresurus stejnegeri*) within Taiwan. *Molecular Ecology* **10**, 1967–1981.
- Culling, M. A., Janko, K., Boroň, A. & Vasilév, V. P. (2006). European colonization by the spiny loach (*Cobitis taenia*) from Ponto-Caspian refugia based on mitochondrial DNA variation. *Molecular Ecology* **15**, 173–190.
- Doadrio, I. & Perdices, A. (2005). Phylogenetic relationships among the Ibero-African cobitids (Cobitis, cobitidae) based on cytochrome *b* sequence data. *Molecular Phylogenetics and Evolution* **37**, 484–493.
- Excoffier, L. & Smouse, P. E. (1994). Using allele frequencies and geographic subdivision to reconstruct gene trees within a species, molecular variance parsimony. *Genetics* **136**, 343–359.
- Excoffier, L., Smouse, P. E. & Quattro, J. (1992). Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial restriction data. *Genetics* **131**, 479–491.
- Fairbanks, R. G. (1989). A 17,000-year glacio-eustatic sea level record: influence of glacial melting rates on the Younger Dryas event and deep-ocean circulation. *Nature* **342**, 637–642.
- Gascoyne, M., Benjamin, G. J., Schwarcz, H. P. & Ford, D. C. (1979). Sea level lowering during the Illinoian glaciation: evidence from a Bahama ‘Blue Bole’. *Nature* **205**, 806–808.
- Hall, T. A. (1999). BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series* **41**, 95–98.
- Hey, J. & Nielsen, R. (2004). Multilocus methods for estimating population size, migration rates, and divergence time, with applications to the divergence of *Drosophila pseudoobscura* and *D. persimilis*. *Genetics* **167**, 747–760.

- Hikida, T. & Ota, H. (1997). Biogeography of reptiles in the subtropical East Asia Island. In *Proceedings of the Symposium on the Phylogeny, Biogeography and Conservation of Fauna and Flora of East Asia Region* (Lue, K. Y. & Chen, T. H., eds), pp. 11–28. Taipei: National Science Council.
- Ho, C. S. (1986). A synthesis of the geologic evolution of Taiwan. *Tectonophysics* **125**, 1–16.
- Hsu, V. (1990). Seismicity and tectonics of a continental-island arc collision zone at the island of Taiwan. *Journal of Geophysical Research* **95**, 4725–4734.
- Huang, C. Y., Yuan, P. B., Song, S. R., Lin, C. W., Wang, C. S., Chen, M. T., Shyu, C. T. & Karp, B. (1995). Tectonics of short-lived intra-arc basins in the arc-continent collision terrane of the Coastal Range, eastern Taiwan. *Tectonics* **14**, 19–38.
- Huelsenbeck, J. P. & Ronquist, F. (2001). MRBAYES: Bayesian inference of phylogeny. *Bioinformatics* **17**, 754–755.
- Kozak, K. H., Blaine, R. A. & Larson, A. (2006). Gene lineages and eastern North American palaeodrainage basins: phylogeography and speciation in salamanders of the *Eurycea bislineata* species complex. *Molecular Ecology* **15**, 191–207.
- Liao, T. Y., Wang, T. Y., Lin, H. D., Shen, S. C. & Tzeng, C. S. (2008). Phylogeography of the endangered species, *Sinogastromyzon puliensis* (Cypriniformes: Balitoridae), in southwestern Taiwan based on mtDNA. *Zoological Studies* **47**, 383–392.
- Lin, C. C. (1957). Topography in Taiwan. *Taiwan Documents* **1**, 67–79.
- Lin, C. C. (1966). An outline of Taiwan's Quaternary geology with a special discussion of the relation between natural history and cultural history in Taiwan. *Bulletin of the Department of Archaeology and Anthropology* **23**, 7–44.
- Lin, C. S. (1990). Distribution of sSphingid moths. *Journal of Taiwan Museum* **43**, 41–94.
- Lin, S. M., Chen, C. A. & Lue, K. Y. (2002). Molecular phylogeny and biogeography of the grass lizards genus *Takydromus* (Reptilia: Lacertidae) of East Asia. *Molecular Phylogenetics and Evolution* **22**, 276–288.
- Liu, T. K., Chen, Y. G., Chen, W. S. & Jiang, S. H. (2000). Rates of cooling and denudation of the early Penglai Orogeny, Taiwan, as assessed by fission-track constraints. *Tectonophysics* **320**, 69–82.
- Nei, M. (1987). *Molecular Evolutionary Genetics*. New York, NY: Columbia University Press.
- Nei, M. & Tajima, F. (1983). Maximum likelihood estimation of the number of nucleotide substitutions from restriction sites data. *Genetics* **105**, 207–217.
- Nielsen, R. & Wakeley, J. (2001). Distinguishing migration from isolation: a Markov chain Monte Carlo approach. *Genetics* **158**, 885–896.
- Oshima, M. (1923). Studies on the distribution of freshwater fishes of Taiwan and discuss the geographical relationships of Taiwan Island and adjacent area. *Zoological Magazine* **35**, 1–49.
- Ota, H. (1991). Systematics and biogeography of terrestrial reptiles of Taiwan. In *Proceedings of the First International Symposium on Wildlife Conservation, ROC* (Lin, Y.-S. & Chang, K.-H., eds), pp. 47–112. Taipei: Council of Agriculture.
- Ota, H. (1997). Historical biogeographical implications in the variation and diversity of amphibians and reptiles in Taiwan. In *Proceedings of the Symposium on the Phylogeny, Biogeography and Conservation of Fauna and Flora of East Asian Region* (Kue K.-Y., Chen T. H., eds), pp. 75–86. Taipei: National Science Council, ROC.
- Page, B. M. & Suppe, J. (1981). The Pliocene Lichi Mélange of Taiwan: its plate tectonic and olistostromal origin. *American Journal of Science* **281**, 193–227.
- Pearse, D. E. & Crandall, K. (2004). Beyond F_{ST} : analysis of population genetic data for conservation. *Conservation Genetics* **5**, 585–602.
- Perdices, A. & Doadrio, I. (2001). The molecular systematic and biogeography of the European cobitids based on mitochondrial DNA sequences. *Molecular Phylogenetics and Evolution* **19**, 468–478.
- Posada, D. & Buckley, T. R. (2004). Model selection and model averaging in phylogenetics: advantages of Akaike Information Criterion and Bayesian approaches over likelihood ratio tests. *Systematic Biology* **53**, 793–808.
- Posada, D. & Crandall, K. A. (1998). MODELTEST: testing the model of DNA substitution. *Bioinformatics* **14**, 817–818.

- Rozas, J., Sanchez-DelBarrio, J. C., Messeguer, X. & Rozas, R. (2003). DnaSP, DNA polymorphism analyses by the coalescent and other methods. *Bioinformatics* **19**, 2496–2497.
- Shen, C. F. (1994). Introduction to the flora of Taiwan. 2. Geotectonic evolution, paleogeography, and the origin of the flora. In *Flora of Taiwan*, 2nd edn, Vol. 1 (Huang, T. C., ed.), pp. 3–7. Taipei: Editorial Committee of the Flora of Taiwan.
- Shih, H. T., Ng, P. K. L. & Chang, H. W. (2004). Systematics of the genus *Geothelphusa* (Crustacea: Decapoda, Brachyura, Potamidae) from southern Taiwan: a molecular appraisal. *Zoological Studies* **43**, 519–526.
- Shih, H. T., Hung, H. C., Schubart, C. D., Chen, C. A. & Chang, H. W. (2006). Intraspecific genetic diversity of the endemic freshwater crab *Candidiopotamon rathbunae* (Decapoda, Brachyura, Potamidae) reflects five million years of the geological history of Taiwan. *Journal of Biogeography* **33**, 980–989.
- Su, H. J. (1992). A geographical data organization system for the botanical inventory of Taiwan. In *Phytogeography and Botanical Inventory of Taiwan* (Peng, C. I., ed.), pp. 23–36. Taipei: Institute of Botany, Academia Sinica Monograph Series.
- Tamura, K., Dudley, J., Nei, M. & Kumar, S. (2007). MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Molecular Biology and Evolution* **24**, 1596–1599.
- Tang, Q., Liu, H., Mayden, R. & Xiong, B. (2006). Comparison of evolutionary rates in the mitochondrial DNA cytochrome *b* gene and control region and their implications for phylogeny of the Cobitoidea (Teleostei: Cypriniformes). *Molecular Phylogenetics and Evolution* **39**, 347–357.
- Tao, H. J. & Hu, C. H. (2001). Cyprinidae fossil fishes of Tainan Hsien, Taiwan. *Ti-Chih (Taipei)* **21**, 51–64 (in Chinese).
- Templeton, A. R. (1993). The ‘Eve’ hypothesis: a genetic critique and reanalysis. *American Anthropologist* **95**, 51–72.
- Teng, L. S. (1990). Geotectonic evolution of late Cenozoic arccontinent collision in Taiwan. *Tectonophysics* **183**, 57–76.
- Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F. & Higgins, D. G. (1997). The Clustal X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acid Research* **24**, 4876–4882.
- Toda, M., Nishida, M., Matsui, M., Lue, K. Y. & Ota, H. (1997). Genetic variation in the Indian rice frog, *Rana limnochairs* (Amphibia: Anura), in Taiwan, as revealed by allozyme data. *Herpetologica* **54**, 73–82.
- Toda, M., Nishida, M., Matsui, M., Wu, G. F. & Ota, H. (1998). Allozyme variation among East Asian populations of the Indian Rice Frog, *Rana limnochairs* (Amphibia: Anura). *Biochemical Systematics and Ecology* **25**, 143–159.
- Tzeng, C. S. (1986). Distribution of the freshwater fishes of Taiwan. *Journal of Taiwan Museum* **39**, 127–146.
- Wang, H. Y., Tsai, M. P., Yu, M. J. & Lee, S. C. (1999). Influence of glaciation on divergence patterns of the endemic minnow, *Zacco pachycaphalus*, in Taiwan. *Molecular Ecology* **8**, 1879–1888.
- Wang, J. P., Hsu, K. C. & Chiang, T. Y. (2000). Mitochondrial DNA phylogeography of *Acrossocheilus paradoxus* (Cyprinidae) in Taiwan. *Molecular Ecology* **9**, 1483–1494.
- Wang, J. P., Lin, H. D., Huang, S., Pan, C. H., Chen, X. L. & Chiang, T. Y. (2004). Phylogeography of *Varicorhinus barbatulus* (Cyprinidae) in Taiwan based on nucleotide variation of mtDNA and allozymes. *Molecular Phylogenetics and Evolution* **32**, 1143–1156.
- Xia, X. & Xie, Z. (2001). DAMBE: data analysis in molecular biology and evolution. *Journal of Heredity* **92**, 371–373.
- Xiao, W., Zhang, Y. & Liu, H. (2001). Molecular systematics of Xenocyprinae (Teleostei: Cyprinidae): taxonomy, biogeography, and coevolution of a special group restricted in East Asia. *Molecular Phylogenetics and Evolution* **18**, 163–173.
- Yang, Y. J., Lin, Y. S., Wu, J. L. & Hui, C. F. (1994). Variation in mitochondrial DNA and population structure of the Taipei tree frog *Rhacophorus taipeianus* in Taiwan. *Molecular Ecology* **3**, 219–228.

- Yu, H. T. (1995). Patterns of diversification and genetic population structure of small mammals in Taiwan. *Biological Journal of the Linnean Society* **55**, 69–89.

Electronic References

- Drummond, A. J. & Rambaut, A. (2005). *BEAST, Version 1.3*. Oxford: Oxford University. Available at http://beast.bio.ed.ac.uk/Main_Page
- Rambaut, A. & Drummond, A. J. (2004). *TRACER, Version 1.2*. Oxford: Oxford University. Available at <http://tree.bio.ed.ac.uk/software/tracer/>
- Schneider, S., Roessli, D. & Excoffier, L. (2000). *Arlequin (Vers. 2.000): A Software for Population Genetic Data Analysis*. Switzerland: Genetics and Biometry Laboratory, University of Geneva. Available at <http://anthro.unige.ch/software/arlequin/>