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Mitochondrial DNA phylogeography of *Glyptothorax fokiensis* and *Glyptothorax hainanensis* in Asia

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Whole mitochondrial DNA cytochrome *b* sequences in 62 fish from 13 locations in Southeast China identified two major clades corresponding to two allopatric taxa, *Glyptothorax fokiensis fokiensis* and *Glyptothorax fokiensis hainanensis*. Reciprocal monophyly and a molecular clock separation between these two taxa of 2·3 million years indicate these taxa should be elevated to species. Mismatch distributions and Fu's F_S statistic suggest that both *G. fokiensis* and *G. hainanensis* have experienced recent population expansions. Analysis of molecular variance indicates that most of the genetic variation resides among populations within both species, with $\Phi_{ST} = 0{\cdot}645$ for *G. fokiensis* and 0·801 for *G. hainanensis*, suggesting restricted gene flow among populations. Significant correlations between the geographic and the genetic distances provide support for the importance of geographic isolations between populations. Nested clade analysis also confirms low levels of genetic exchanges between the two major groups and between populations within each group. The phylogeographical pattern among populations of *Glyptothorax* in East Asia can be attributed to historical fragmentations, demographic expansions and occasional long-distance dispersals stimulated by tectonic activity and Ice Age climate changes.

Key words: *Glyptothorax fokiensis*; *Glyptothorax hainanensis*; mtDNA cytochrome *b*; phylogeography; population structure; speciation.

INTRODUCTION

The Southeast Asian freshwater fish fauna represents the richest ichthyofauna in the Sino-Indian region (Banarescu, 1991). Freshwater fish richness reflects the geographical complexity of Asia, which is subdivided into South, East and Central Asia sub-regions (Banarescu, 1991; Banarescu & Coad, 1991). Within East Asia, the sisorid catfish *Glyptothorax fokiensis* Rendahl, 1925

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species group (Sisoridae) offers an excellent opportunity to reconstruct the phylogeographical structure of populations over a large geographical region. The freshwater fish *Glyptothorax fokiensis sensu lato* inhabits the bottom of streams and fast-flowing water. This taxon consists of allopatric population groups designated as three sub-species: *G. fokiensis fokiensis* Rendahl, 1925, *Glyptothorax fokiensis hainanensis* Nichols & Pope, 1927 and *Glyptothorax fokiensis honghensis* Li, 1984 (Li, 1984*a*, *b*; Chu *et al.*, 1999). *Glyptothorax f. honghensis* is distributed in northeastern Laos, the Red River basin in Vietnam, and Yunnan, while *G. f. fokiensis* and *G. f. hainanensis* are distributed in Southeast China. The systematic status of the *G. fokiensis* complex in Southeast China, however, remains uncertain. *Glyptothorax hainanensis* and *G. fokiensis* have been treated as either sister species (Mo & Chu, 1986) or sub-species (Li, 1984*a*, *b*; Chu *et al.*, 1999). Morphologically, vertebral number, 32–34 in *G. f. hainanensis* and 34–37 in *G. f. fokiensis*, differentiates them from each other. Both taxonomic hypotheses indicate a close affinity between the two sister taxa and the existence of biological discontinuities.

Considering geohistorical events and the distributions of ichthyofauna, Li (1981) identified five major geographical regions in China: 1) North region, 2) West China, 3) Mongolia-Ninxia region, 4) East China and 5) South China. Sub-regions were further designated within each of these regions. *Glyptothorax f. fokiensis* occurs in the Yangtze River (Changjiang) sub-region within the East China region and in the Pearl River and ZheMin sub-regions of the South China district, while *Glyptothorax f. hainanensis* is distributed in the Hainan sub-region in the South China region (Chu *et al.*, 1999).

The debate over the criteria to recognize species and sub-species boundaries has received considerable attention (Avise & Walker, 1998). In theory and practice, nearly all populations of a species exhibit genetic differentiation to some degree (Avise & Walker, 1998). Thus, the criteria used to recognize a species, sub-species or any other rank categories often depend on the biological attributes of the taxa in question. Recent studies have used molecular data to define species boundaries, particularly to test existing hypotheses of species-level relationships. For example, recent work on species boundaries of the snake *Pituophis melanoleucus* Stull, 1940 indicates that the traditional view of this single polytypic species is inconsistent with molecular evidence and that the recognition of three distinct species within the complex more accurately reflects the evolutionary history of the group (Rodriguez-Robles & De Jesus-Escobar, 2000). Similar works on the freshwater fishes Xenocyprinae (Xiao *et al.*, 2001) and *Zacco platypus* Temminck & Schlegel, 1846 (Perdices *et al.*, 2004) in Southeast China suggest that tectonic activity during the Cenozoic in Southeast China transformed river trajectories and triggered subsequent speciation and divergence.

This study focuses on two main issues. The first is to investigate the systematic status of the *G. fokiensis* complex, with an emphasis on defining species and sub-species boundaries. The second is to test the historical hypotheses of phylogeographical structure of these freshwater fishes in Southeast China. These questions are addressed by using mitochondrial DNA (mtDNA) cytochrome *b* (cyt *b*) haplotypes to construct a phylogeny and to infer the demographic histories of populations in these taxa.

MATERIALS AND METHODS

SAMPLES AND SEQUENCING

The entire mitochondrial cyt *b* gene was sequenced in 62 specimens from 13 localities across East and South China that represented both putative sub-species (Fig. 1). Two other sisorid catfishes, *Glyptothorax cavia* Hamilton, 1822 (AF477830) and *Pseudecheneis sulcatus* Day, 1889 (AF499601), were used as out-groups. Specimens are lodged in the Department of Biology, South China Normal University. Locality data and sample number are provided in Table I. In the ichthyofauna classification of China (Li, 1981), the 10 streams that were sampled belong to four sub-regions: Yangtze River sub-region (two tributaries of Yangtze River: Guizhou, GZ, and Ganjiang, GJ), Pearl River sub-region (Moyangjiang, MY; three tributaries of Pearl River: Xijiang, XJ; Beijiang, BJ; Dongjiang, DJ), ZheMin sub-region (Minjiang, MJ; Jiulongjiang, JL; Hanjiang, HJ) and Hainan sub-region (Hainan Island: Wanquan River, WQ; Leizhou Peninsula: Lianjiang, LJ; Nanliujiang, NJ; Jianjiang, JJ) (Table I and Fig. 1).

Samples were stored in 100% ethanol. Genomic DNAs were extracted from muscle tissue by a standard protocol of Blin & Stafford (1976). The entire cyt *b* gene was amplified using polymerase chain reactions (PCR) 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 (8 mM), 10 pmol of each primer and 4U

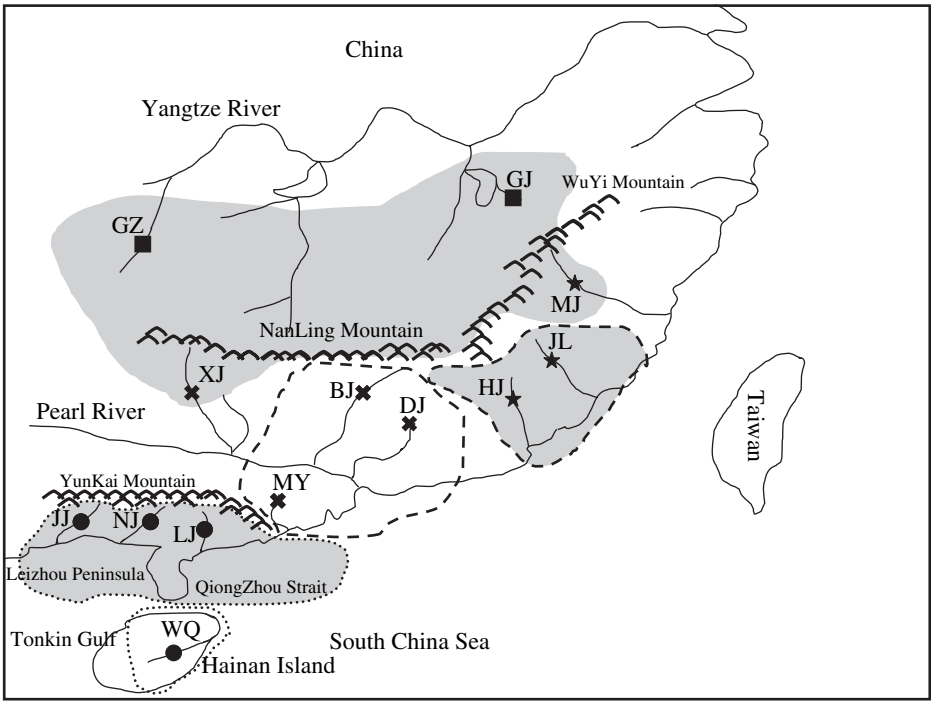

FIG. 1. Distributional ranges of the two nominal sub-species of *Glyptothorax fokiensis* species group in China. Phylogenetic clades with localities labelled as shown in Fig. 2. Sampled location – East China region: ■, Yangtze River sub-region; South China region: ★, ZheMin sub-region; ✖, Pearl River sub-region and ●, Hainan sub-region. , distribution of *G. f. fokiensis* clade I; , distribution of *G. f. fokiensis* clade II; , distribution of *G. f. fokiensis* clade III; , distribution of *G. f. hainanensis* clade α and , distribution of *G. f. hainanensis* clade β.

TABLE I. Sample locations, abbreviations, sample size, number of haplotypes, diversity statistics and tests of neutrality for samples of *Glyptothorax*

Taxa	Population	Abbreviation	Sample size	Number of haplotype	Haplotype diversity (*h*)	Nucleotide diversity (%)		Tajima's *D*	Fu and Li's *D**	Fu's F_s
						Θ_π	Θ_W			
G. f. fokiensis			43	37	0·992	4·062	4·313	−0·21 459	0·13 398	−4·182*
	Guizhou River	GZ	10	7	0·911	0·553	0·745	−1·22 898	−1·60 046	−0·197
	Ganjiang River	GJ	4	4	1·000	0·439	0·479	−0·83 379	−0·83 379	−0·524
	Minjiang River	MJ	3	3	1·000	0·703	0·703	NA	NA	0·901
	Jiulongjiang River	JL	6	5	0·933	0·978	1·232	−1·30 466	−1·35 078	0·689
	Hanjiang River	HJ	4	3	0·833	0·132	0·144	−0·75 445	−0·75 445	−0·288
	Dongjiang River	DJ	4	4	1·000	0·571	0·575	−0·07 004	−0·07 004	−0·187
	Beijiang River	BJ	4	4	1·000	0·659	0·671	−0·18 144	−0·18 144	−0·010
	Xijiang River	XJ	4	4	1·000	0·615	0·671	−0·84 532	−0·84 532	−0·095
	Moyangjiang River	MY	4	3	0·833	0·176	0·192	−0·78 012	−0·78 012	0·134
G. f. hainanensis			19	19	1·000	1·875	2·063	−0·37 800	−0·72 399	−6·371**
	Jianjiang River	JJ	6	6	1·000	0·527	0·616	−0·88 901	−0·91 639	−1·813
	Lianjiang River	LJ	4	4	1·000	0·469	0·527	−1·11 654	−0·83 741	−0·439
	Nanliujiang River	NJ	2	2	1·000	0·088	0·088	NA	NA	NA
	Wanquan River	WQ	7	7	1·000	0·678	0·789	−0·79 456	−0·86 046	−2·053
Total			62	56	0·997	5·978	8·549	−0·08 043	−0·16 926	−9·96

*$P < 0·05$, **$P < 0·01$.

of *Taq* polymerase (Promega, Madison, WI, U.S.A.). 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, Valencia, CA, U.S.A.). Products of the cycle sequencing reactions were run on an ABI 377 automated sequencer (Applied Biosystems, Foster City, CA, U.S.A.). All sequences were deposited in the GenBank database under accession numbers AM235787–AM235847.

DATA ANALYSIS

Nucleotide sequences were aligned with ClustalX 1.81 (Thompson *et al.*, 1997), and alignments were verified by eye. Levels of intra-population genetic diversity were estimated by indices of haplotype diversity (*h*) (Nei & Tajima, 1983) and by nucleotide diversity (Θ_π, Θ_W) (Nei, 1987). Interpopulation genetic diversity was estimated by nucleotide divergence D_a using DnaSP 3.14 (Rozas & Rozas, 1999). The TVM + G model using hierarchical likelihood ratio tests method (Abdo *et al.,* 2005) of DNA substitution was the most appropriate model for the analyses by applying MODELTEST 3.06 (Posada & Crandall, 1998) to the dataset (base frequencies: A, 0·2906; C, 0·2791; G, 0·1361 and T, 0·2942; proportion of invariable sites = 0 and gamma distribution shape parameter = 0·2468). A haplotype genealogy was generated by neighbour joining (NJ) in MEGA2 (Kumar *et al.*, 2001) and by maximum likelihood (ML) in PAUP* 4.0b2 (Swofford, 2002). Bootstrapping was performed with 400 replications for ML trees and 1000 replicates for NJ trees. The number of mutations between DNA haplotypes was calculated using MEGA2 and was used to construct a minimum spanning network with MINSPNET (Excoffier & Smouse, 1994) in a hierarchical manner (Chiang *et al.*, 2001). This network was then converted into a nested design and analysed by GEODIS 2.0 (Posada *et al.*, 2000), with the null hypothesis of no geographical association among haplotypes. The inference key of Templeton (2004) was used to infer processes producing statistically significant associations.

The hypothesis of a molecular clock was tested by a relative rate test (Wu & Li, 1985). Based on the assumption of a normal distribution of nucleotide substitutions (Wu & Li, 1985), the hypothesis of a molecular clock will be rejected with 95% significance when the difference of substitution rates between two lineages is >1·96 times the S.E. (*cf.* Chiang & Schaal, 2000). The total number of nucleotide substitutions (*K*) was calculated for each lineage using a sequence of an out-group species, *G. cavia*. The number and ratio of transversions (tv) to transitions (ti) between sequences were obtained using MEGA2 (Kumar *et al.*, 2001). A molecular clock rate of 2% per million years was assumed (Bermingham *et al.*, 1997; Johns & Avise, 1998). In this study, divergence times between populations in the *G. fokiensis* species group were estimated from the average number of nucleotide substitutions (*K*) among lineages, after removing sequences that deviated from a molecular clock model. Divergence times were then estimated using penalized likelihood, as implemented in rates of evolution analysis (Sanderson, 2002). This semi-parametric method does not assume clocklike molecular evolution but allows different evolutionary rates on each branch. In addition, there is an element of rate autocorrelation in penalized likelihood based on the idea that descendants inherit their evolutionary rate from ancestors. A smoothing value determines the relative importance of the likelihood score and the autocorrelation penalty for the optimality score in penalized likelihood. The optimal smoothing value is determined through a cross-validation procedure (Sanderson, 2002; Wang *et al.*, 2004). A 95% CI was calculated for two relevant nodes using an algorithm in rates of evolution analysis (r8s) (Sanderson, 2002) with a cut off value of 4. At least one dated reference point is needed to calibrate the output from r8s programme into actual age estimates. However, as no fossils from *G. fokiensis* species group are known, the penalized likelihood analysis was performed on a dataset representing divergence between lineages. The two

other sisorid catfishes (*G. cavia* and *P. sulcatus*) were used as out-groups to root the tree. Divergence time between populations in South China was used as a calibration point. The ML method in PHYLIP 3.6a2 (Felsenstein, 1993) was used to estimate branch lengths with the general time reversible model. The best tree recovered was used as input to r8s. An unconstrained penalized likelihood analysis (Sanderson, 2002) was conducted using the Powell algorithm.

Several statistics were used to investigate historical demographics. Mismatch distributions are expected to display a unimodal pattern after a population expands from a single source or recovers from a bottleneck (Slatkin & Hudson, 1991), but a multimodal distribution is expected after long periods of constant population size (Smith *et al.*, 2001). A Poisson distribution of mismatches is expected in recently expanding populations (Slatkin & Hudson, 1991). DnaSP 3.99 (Rozas & Rozas, 1999) was used to compute D (Tajima, 1989*a*), D^* (Fu, 1997) and F_S (Fu, 1997) to assess departures from neutrality (Tajima, 1989*b*) in the individual population samples. Ramos-Onsins & Rozas (2002) suggested that F_S has greater power to detect population growth with moderate sample sizes than the other estimators. DnaSP was also used to estimate the time since the population expansion from the pair-wise mismatch distribution.

RESULTS

SEQUENCE VARIATION

A total of 62 complete cyt *b* sequences were obtained from 13 populations of *G. f. fokiensis* and *G. f. hainanensis* and two out-group taxa, *G. cavia* and *P. sulcatus*. A total of 1138 bp were amplified, with 368 sites variable in the total dataset (including both out-groups), of which 230 were parsimoniously informative. A total of 254 variable sites (204 parsimoniously informative) appeared among sequences of the *G. fokiensis* species group. Nucleotide sequences of cyt *b* in the *G. fokiensis* species group were rich in A + T (58·7%), as in many other fishes (Johns & Avise, 1998). Base compositions of A = 0·284, C = 0·275, G = 0·138 and T = 0·303 overall and of A = 0·322, C = 0·258, G = 0·127 and T = 0·292 in the third-codon positions, indicate a strong bias against guanine, a result consistent with most vertebrate mitochondrial genomes (Zhang & Hewitt, 1996) and with fish cyt *b* sequences in particular (Johns & Avise, 1998). Nucleotide substitutions were biased toward transitions and with a mean ti:tv ratio of 4·1. This level of transition bias is within the range of biases previously reported for vertebrates (Meyer, 1993; Avise, 2004).

Pair-wise sequence divergences (D_a) between sequences of *G. fokiensis* species group and two out-groups ranged from 6·22 to 22·49% (Table II). Maximum divergence between cyt *b* sequences within haplotype groups ranged from 4·08 (*G. f. hainanensis*) to 7·19% (*G. f. fokiensis*) (Table III). Considering all samples together, maximum divergence was 9·70%. Sequence divergences between the two sub-species haplotype groups ranged from 6·22 to 9·70%, with an average of 8·03%, whereas divergences between the in-groups and the two out-groups ranged from 13·91 to 21·39%. Divergences between *Glyptothorax* and *Pseudecheneis* ranged from 19·72 to 21·39%.

MTDNA GENE GENEALOGY

The topologies of the NJ and the ML trees were identical, with only small differences in bootstrap values. Thirty-seven cyt *b* haplotypes in *G. f. fokiensis*

TABLE II. Pair-wise differences (uncorrected per cent divergence) within and among haplotype groups for mitochondrial cytochrome *b*. Average (minimum–maximum) per cent divergences among haplotype groups appear above the diagonal and per cent divergences within haplotype groups appear along the diagonal in bold

	G. f. fokiensis	*G. f. hainanensis*	*G. fokiensis*	*G. cavia*	*Pseudechen* sp.
G. f. fokiensis	**4·23 (0·10–7·19)**	8·03 (6·22–9·70)		13·91 (13·20–14·74)	21·39 (19·72–22·49)
G. f. hainanensis		**1·91 (0·10–4·08)**		14·46 (13·85–15·26)	20·32 (18·39–20·87)
G. fokiensis			**5·64 (0·10–9·70)**	14·08 (13·20–15·26)	21·06 (18·39–22·49)
G. cavia					19·72

and 19 in *G. f. hainanensis* fell into two major clades, respectively, in the consensus NJ tree, with significant bootstrap support (Fig. 2). Within *G. f. fokiensis*, three clades were identified: clade I [[(GZ, MJ), XJ], GJ] (bootstrap support of 61%), clade II [(BJ, DJ), MY] (bootstrap 57%) and clade III (HJ, JL) (bootstrap 97%) (Fig. 2). Within clade I, population GJ in the upper reaches of the Changjiang River appeared in a basal position in the tree. The Xijiang population (XJ) in the west Pearl River sub-region and the Minjiang population (MJ) in the north ZheMin sub-region were nested within a cluster, containing populations of the Changjiang sub-region (GZ and GJ). Within clade II, which was restricted to the east Pearl River region, the monophyletic group, BJ and DJ, was a sister group to the population MY. These two lineages were separated into two independent rivers, the Pearl and Moyangjiang rivers, respectively. Within clade III, two populations HJ and JL are distributed in rivers in the south ZheMin sub-region (Figs 1 and 2).

TABLE III. Pair-wise F_{ST} (below diagonal) and per cent nucleotide divergence (above diagonal) between populations based on mtDNA

	GZ	GJ	MJ	JL	HJ	DJ	BJ	XJ	MY	JJ	LJ	NJ	WQ
GZ		3·943	2·358	5·195	4·649	4·633	4·279	3·135	4·163	7·440	7·069	7·425	7·032
GJ	0·874		3·603	6·136	5·426	4·855	4·635	4·031	4·921	8·165	7·799	7·975	7·949
MJ	0·733	0·841		4·823	4·723	4·211	3·837	2·768	3·962	7·352	6·810	7·337	6·950
JL	0·852	0·884	0·825		3·354	5·320	5·602	5·045	6·016	8·126	7·894	7·967	8·009
HJ	0·926	0·947	0·911	0·834		5·163	5·272	5·053	5·822	8·048	7·667	8·018	7·774
DJ	0·878	0·895	0·848	0·854	0·931		1·406	4·514	4·416	8·172	7·634	8·348	7·783
BJ	0·858	0·881	0·822	0·853	0·925	0·562		4·558	3·932	7·975	7·436	8·150	7·435
XJ	0·813	0·869	0·761	0·842	0·926	0·868	0·860		4·438	6·810	6·415	6·898	6·553
MY	0·912	0·937	0·889	0·904	0·973	0·915	0·893	0·910		7·996	7·469	7·996	7·344
JJ	0·927	0·940	0·916	0·907	0·959	0·932	0·925	0·916	0·956		2·314	1·172	2·699
LJ	0·927	0·941	0·913	0·908	0·960	0·931	0·924	0·915	0·956	0·784		2·417	1·955
NJ	0·956	0·966	0·946	0·933	0·986	0·960	0·954	0·949	0·983	0·737	0·884		2·730
WQ	0·912	0·929	0·900	0·896	0·947	0·919	0·910	0·901	0·941	0·776	0·706	0·859	

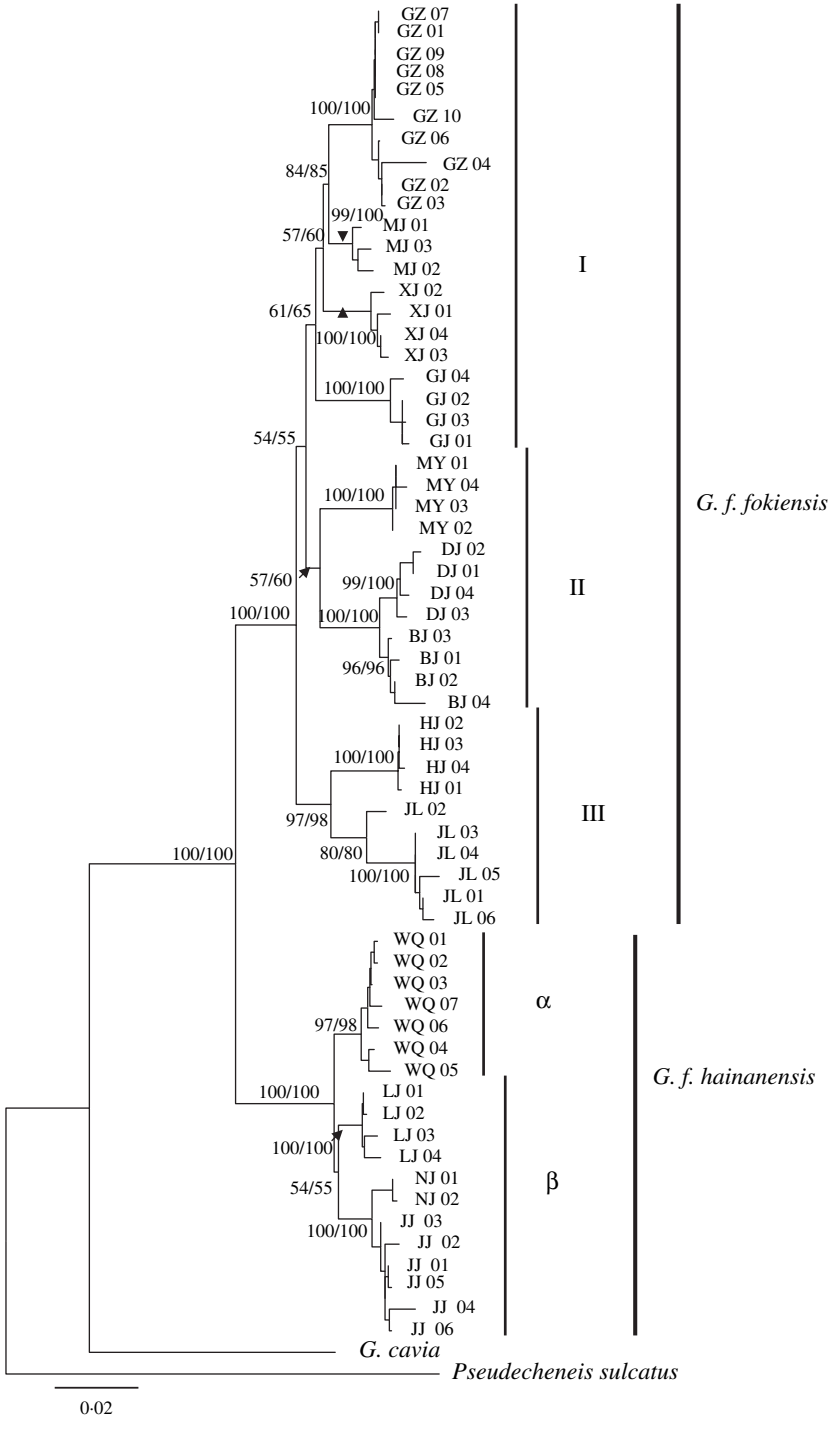

FIG. 2. Rooted NJ bootstrap tree of mtDNA haplotypes in the *Glyptothorax fokiensis* species group. Bootstrap values above branches for NJ (left) and ML (right) algorithms.

The topology of the minimum spanning network of the *G. fokiensis* species group largely agreed with that of the NJ tree (Figs 3 and 4). Haplotypes from a given population were monophyletic. Within *G. f. fokiensis*, three disjoined clades (2-1, 2-2 and 2-3) were observed (Fig. 3), corresponding to lineages, I, II and III, respectively. Clade 2-2 (DJ, BJ and MY) formed an interior clade linked to clades 2-1 and 2-3. The nested clade analysis suggests that the geographical associations observed in DJ, BJ and MY (clade 2-2) can be explained by long-distance colonization. However, no conclusive result for clade 2-1 was observed. It was not possible to distinguish between past fragmentation and long-distance colonization [Table IV(a, b)].

Two clades were recognized within *G. f. hainanensis*, α (WQ) (bootstrap support 97%) and β [(NJ, JJ), LJ] (bootstrap 54%), which correspond to Hainan Island and Leizhou Peninsula, respectively, in the Hainan sub-region (Fig. 2). In the haplotype network, four clades were identified, 1-A (JJ), 1-B (NJ), 1-C (LJ) and 1-D (WQ) (Fig. 4). The first two clades were nested in a higher level clade (2-A). Three disjoined clades (2-A, 1-C and 1-D) in the network corresponded to three geographical regions, west Leizhou Peninsula, east Leizhou Peninsula and Hainan Island, respectively. The inference key suggested allopatric fragmentation for clades 2-A and for the entire cladogram [Table V(a, b)].

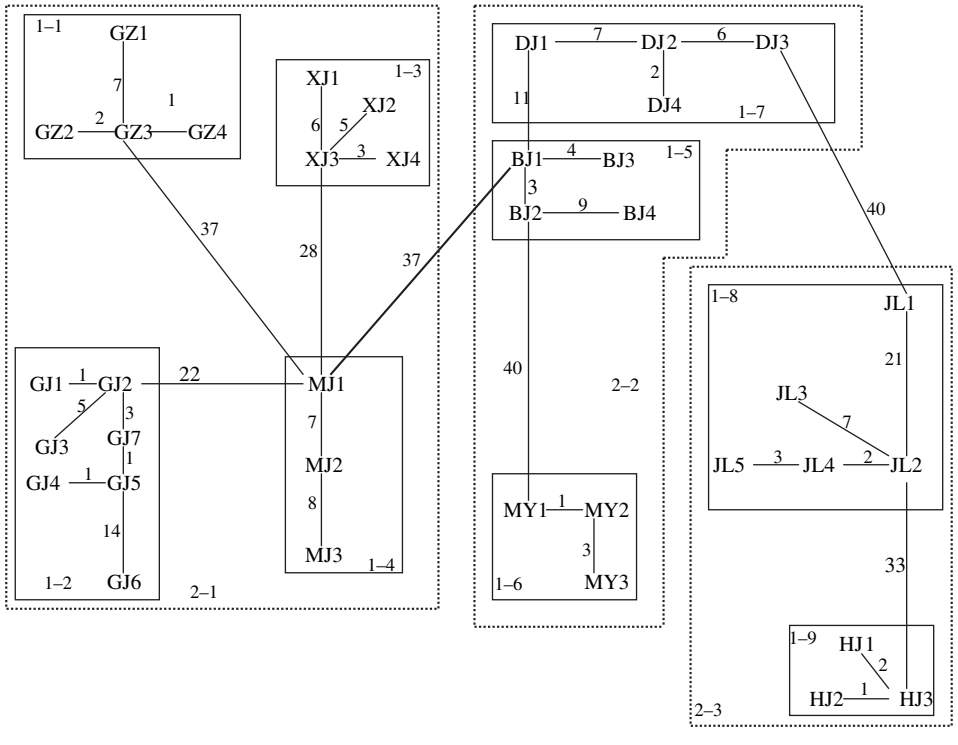

FIG. 3. Minimum spanning network of mtDNA cytochrome *b* in *Glyptothorax fokiensis* populations. Numbers at nodes indicate number of nucleotide changes between haplotypes.

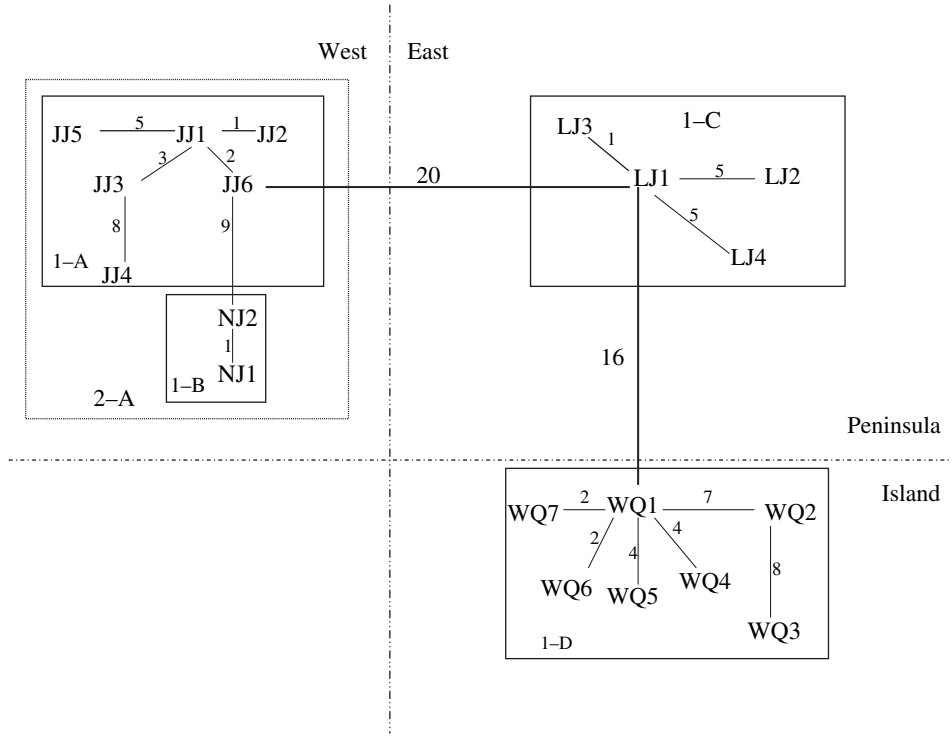

FIG. 4. Minimum spanning network of mtDNA cytochrome *b* haplotypes in populations of *Glyptothorax hainanensis*. See Fig. 3 for details.

MOLECULAR DATING

The relative rate tests between mtDNA sequences, with *G. cavia* as an outgroup, revealed that most lineages were consistent with a molecular clock. However, the lineage leading to DJ04 appears to have evolved relatively rapidly. After removing this exception, the number of nucleotide substitutions (*K*) between haplotypes averaged 0·041. Divergence between *G. f. fokiensis* and *G. f. hainanensis* was dated to 2·3 million years ago (MYA). After calibration of the node between the two sub-species, the evolutionary rate was estimated to be 2·05% changes per million years in the cyt *b* gene with the r8s programme. Within *G. f. fokiensis*, the time of divergence between the East China populations (GZ and GJ) and the South China populations (MJ, XJ, DJ, BJ, JL, HJ and MY) was estimated to be 1·10 MYA in the early Pleistocene. Within *G. f. hainanensis*, divergence between Hainan Island and Leizhou Peninsula populations was estimated to be 0·72 MYA.

GENETIC VARIATION WITHIN AND AMONG POPULATIONS

Haplotype diversities (*h*) within populations of *G. f. fokiensis* were large, ranging between 0·833 (MY) and 1·00 (GJ, MJ, DJ, BJ and XJ) (Table I). Hierarchical calculations revealed that the nucleotide divergences among areas

TABLE IVa. Nested clade analysis of mtDNA haplotypes in *Glyptothorax fokiensis fokiensis*. Dc and Dn are clade and nested distances, respectively. Superscript letters indicate significantly smaller (S) or larger (L) than expected geographical distances between clades

One step	Dc	Dn	Two step	Dc	Dn
$1\text{-}1_T$	$0{\cdot}00^S$	$749{\cdot}46^L$	$2\text{-}1_T$	617·61	$663{\cdot}92^L$
$1\text{-}2_T$	$0{\cdot}00^S$	543·03			
$1\text{-}3_T$	$0{\cdot}00^S$	537·57			
$1\text{-}4_I$	0·00	$753{\cdot}16^L$			
I/T	0·00	$165{\cdot}47^L$			
$1\text{-}5_I$	$0{\cdot}00^S$	180·21	$2\text{-}2_I$	$221{\cdot}19^S$	$494{\cdot}46^S$
$1\text{-}6_T$	$0{\cdot}00^S$	$293{\cdot}89^L$			
$1\text{-}7_I$	$0{\cdot}00^S$	189·47			
I/T	$0{\cdot}00^S$	$-109{\cdot}0^S$			
$1\text{-}8_I$	$0{\cdot}00^S$	55·38	$2\text{-}3_T$	$62{\cdot}60^S$	533·27
$1\text{-}9_T$	0·00	$72{\cdot}00^L$			
I/T	0·00	$-16{\cdot}61^S$	I/T	$158{\cdot}58^L$	33·95

Subscript letter T = Tip clade, I = Interior clade.

TABLE IVb. Inferences from significant tests of nested clade analysis of mtDNA haplotypes in *Glyptothorax fokiensis fokiensis*

Clade	Permutational χ^2	*P*	Clade key	Inferences
One-step clades nested in 2-1	63·00	0·000	1-2-3-5-15-NO	Past fragmentation or long-distance colonization
One-step clades nested in 2-2	24·00	0·000	1-2-11-12-13-YES	Long-distance colonization possibly coupled with subsequent
One-step clades nested in 2-3	10·00	0·004	1-19-NO	Allopatric fragmentation
Whole cladogram	86·00	0·000	1-2-11-17-4-9-10-YES	Allopatric fragmentation

(with an average of 4·582%) was larger than those between populations (mean = 4·450%) (Table III). Among the regions, the nucleotide divergence between MY (Pearl River sub-region) and HJ (ZheMin sub-region) (5·822%) was the most diverged among comparisons (Table III). Within sub-regions, nucleotide diversity was large in the populations of the Pearl River (mean = 0·618%), ZheMin (mean = 0·604%) and Changjiang (mean = 0·496%) sub-regions. Within-population nucleotide diversity was largest in population JL of the ZheMin sub-region (0·978%) and smallest in population HJ of the ZheMin sub-region (0·132%) (Table I). Hierarchical analyses of sequence differences with AMOVA indicated that a substantial proportion of the molecular variance was attributable to differences among populations ($\Phi_{ST} = 0{\cdot}645$, $P < 0{\cdot}001$) and to

TABLE Va. Nested clade analysis of mtDNA haplotypes in *Glyptothorax fokiensis hainanensis*. Dc and Dn are clade and nested distances, respectively. Superscript letters indicate significantly smaller (S) or larger (L) than expected geographical distances between clades

One-step	Dc	Dn	Two-step	Dc	Dn
$1\text{-}A_I$	$0{\cdot}00^S$	$10{\cdot}22^S$	$2\text{-}A_T$	$13{\cdot}80^S$	174·68
$1\text{-}B_T$	0·00	$21{\cdot}23^L$			
I/T	0·00	$-11{\cdot}00^S$			
			$1\text{-}C_I$	$0{\cdot}00^S$	167·94
			$1\text{-}D_T$	$0{\cdot}00^S$	258·40
			I/T	−7·36	$-45{\cdot}81^S$

Subscript letter T = Tip clade, I = Interior clade.

TABLE Vb. Inferences from significant tests of nested clade analysis of mtDNA haplotypes in *Glyptothorax fokiensis hainanensis*

Clade	Permutational χ^2	*P*	Clade key	Inferences
One-step clades nested in 2-A	8·00	0·037	1-19-NO	Allopatric fragmentation
Whole cladogram	37·00	0·000	1-19-NO	Allopatric fragmentation

differences among populations within regions ($\Phi_{SC} = 0{\cdot}492$; $P = 0{\cdot}009$). The relative contribution of differences among regions was small ($\Phi_{CT} = 0{\cdot}0161$, $P = 0{\cdot}07$). Geographical subdivision assessed by DnaSP (Table III) indicated low levels of differentiation among populations within regions. F_{ST} averaged 0·874 in the Changjiang sub-region, ranged from 0·825 to 0·911 in the ZheMin sub-region and from 0·562 to 0·868 in the Pearl River sub-region. In contrast, higher levels of differentiation existed between populations in other regions, with F_{ST} ranging from 0·733 between GZ and MJ to 0·973 between HJ and MY. After pooling by region, the smallest comparison was $F_{ST} = 0{\cdot}862$ between the ZheMin and the Pearl River sub-regions.

Haplotype diversity is also remarkably high within populations of *G. f. hainanensis* (Table I). Hierarchical calculations revealed that nucleotide divergence between areas (mean $D_a = 2{\cdot}461\%$) was greater than divergences between populations within areas (mean $D_a = 2{\cdot}215\%$) (Table III). At the inter-region level, nucleotide divergence between populations on Leizhou Peninsula and Hainan Island varied from 1·955 to 2·730%. Within regions, nucleotide diversity was large in the population on Hainan Island (0·789%) and small in the populations on Leizhou Peninsula (mean = 0·410%) (Table I). Within-population nucleotide diversity was greatest in population WQ in Hainan Island (0·789%) and least in population LJ in Leizhou Peninsula (0·527%) (Table I). AMOVA indicated that the proportion of molecular variance was attributable to differences among populations ($\Phi_{ST} = 0{\cdot}801$, $P < 0{\cdot}001$). The relative contribution of differences among regions was also large ($\Phi_{CT} = 0{\cdot}724$, $P < 0{\cdot}001$). Differentiation among populations within regions ($F_{ST} = 0{\cdot}802$ within

Leizhou Peninsula) was greater than that among populations in different regions ($F_{ST} = 0{\cdot}780$) (Table III).

POPULATION DEMOGRAPHY

Mismatch distributions were multimodal and ragged in both *G. f. fokiensis* and *G. f. hainanensis* (Fig. 5). Mismatch peaks close to the origin represent variation within populations, whereas those to the right represent differences among populations. The mean number of differences between sequences of *G. f. fokiensis* [(46·14); Fig. 5(a)] was twice the mean for *G. f. hainanensis* [(21·32); Fig. 5(b)]. Estimates of *Nm* (number of immigrants per generation) were 0·07 and 0·12 for *Glyptothorax f. fokiensis* and *G. f. hainanensis*, respectively. These small *Nm* values fit the predicted distribution under expansion models (Barrowclough *et al.*, 1999; Ray *et al.*, 2003). Values of Tajima's *D*

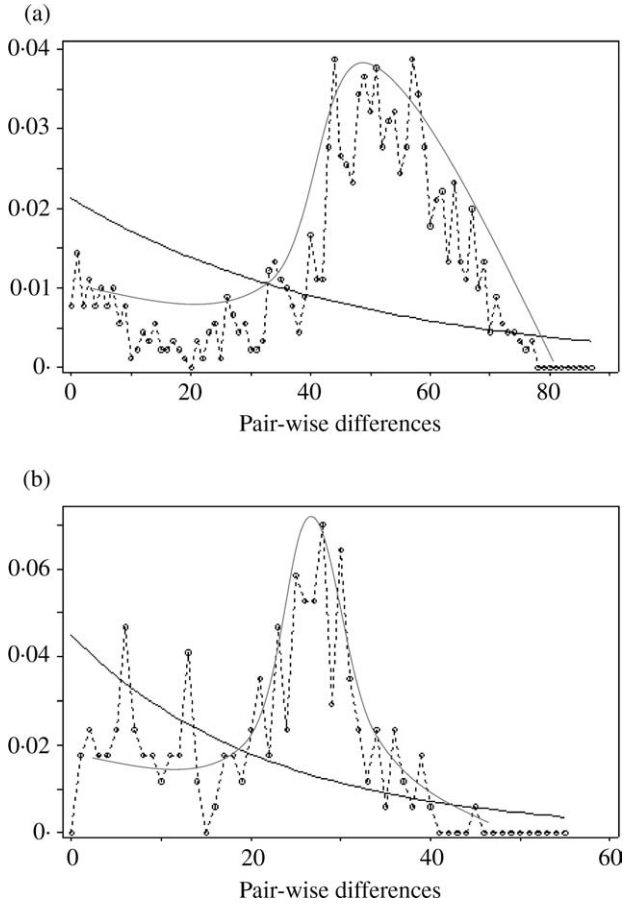

FIG. 5. Mismatch distributions corresponding to (a) *Glyptothorax fokiensis* and (b) *Glyptothorax hainanensis*. Solid lines represent the distribution expected under constant population size and dotted lines represent the observed distribution. The theoretical expected curve for a recent population expansion is grey.

for *G. f. fokiensis* and *G. f. hainanensis* were negative but were not significantly different from zero (Table I).

DISCUSSION

SPECIES STATUS OF *G. FOKIENSIS* COMPLEX

This study examined the species status of the *G. fokiensis* species group. Two major mtDNA clades, corresponding to the nominal sub-species of *G. f. fokiensis* and *G. f. hainanensis*, were recovered. Are the taxonomic ranks of these taxa consistent with estimates of sequence divergence? Johns & Avise (1998) calculated and compared the levels of cyt *b* sequence divergence between sister species, congeneric species and confamilial genera within and across the major vertebrate taxonomic classes. Some salient trends emerge from these summaries. Divergences between 81 confamilial fish genera averaged 5·6% (Johns & Avise, 1998). In the present study, divergences (D_a) both between and within taxa of *G. fokiensis* ranged from 6·22 to 9·70% and are much larger than estimates of intraspecific divergences in other species. These large sequence divergences support the hypothesis that the two major taxa within *Glyptothorax* should be elevated to species, *G. fokiensis* and *G. hainanensis*. Molecular dating suggested that divergence between *G. fokiensis* and *G. hainanensis* began about 2·3 MYA, at a time corresponding to the separation between northern and southern geographical regions by the YunKai Mountains (Fig. 1). Geographical isolation between ancestral *G. fokiensis* and *G. hainanensis* most probably triggered speciation. These taxa now show substantial genetic divergence from each other, and each contains unique haplotypes reflecting a long period of lineage sorting.

GENETIC POPULATION STRUCTURE AND PHYLOGEOGRAPHY OF *G. FOKIENSIS*

The mtDNA gene genealogy of *G. fokiensis* exhibited significant geographical structuring of three mtDNA lineages. Clade I contained four populations belonging to regions in East and South China, including the Yangtze River, west Pearl River and north ZheMin sub-regions. The two other clades were restricted to the South China region (Fig. 1). They included populations in the eastern Pearl River sub-region (clade II) and the southern ZheMin sub-region (clade III). The AMOVA indicated that regional structuring of the three lineages explained much of the total genetic variance. The large sequence divergences between clades indicate that they have been isolated from one another for a considerable amount of time.

Within clade I, the close phylogenetic relationship between the populations Guizhou and Minjiang in the upper reaches of Yangtze River was unexpected (Fig. 2). These populations are separated by a much greater distance than are the Guizhou and Minjiang populations (Fig. 1). These findings are consistent with the hypotheses of Liu (1993), based on the phylogeny of Sinipercine and of Xiao *et al.* (2001), based on systematic analyses of molecular characters and parasite infections, in indicating a close biogeographical affinity between

populations in the Yangtze and Minjiang rivers. Furthermore, nested clade analysis indicated that the populations Guizhou and Ganjiang in Yangtze River tributaries are genetically distinct from each other. The upper Yangtze River is separated from the lower reach of the river by Three Gorges, an apparent barrier to migration that also separates populations of Xenocyprinae (Xiao *et al.*, 2001) and *Z. platypus* Temminck & Schlegel, 1846 (Perdices *et al.*, 2004).

Cenozoic tectonic movements produced topographical complexity in Southeast Asia that transformed river trajectories (Rainboth, 1991). This geographical complexity has also been important in preventing gene flow between north and south populations of some Asiatic cyprinids (Perdices *et al.*, 2004). The Xijiang population in the upper Pearl River is more closely related to populations in the Minjiang and Yangtze rivers than to those in the middle reaches of Pearl River (Fig. 1). Nevertheless, these drainages are currently unconnected. The genetic similarity may be due to historical flows of rivers in the eastern plain of China, which allowed exchanges of the fish fauna among rivers (*cf.* Chen *et al.*, 1986). Moreover, the NanLing Mountains, which have an east-west orientation, represent prominent watersheds and north-south dispersal barriers. The WuYi Mountains have a north-south orientation and block east-west dispersals between populations on either side of these mountains. Connections between populations of East China (clade 1-1 and 1-2) and South China in the mtDNA network and the interior position of Minjiang (clade 1-4) may indicate that Minjiang represents a source population for the colonization of *G. fokiensis* in East China. Furthermore, the splitting between eastern and southern regions (clades 1-1, 1-2 and 1-4) may be to subdivision by the elevation of WuYi Mountains (Fig. 1), a vicariant event occurring about 1·10 MYA.

The interior position of clade 2-2 in the minimum spanning network and higher frequencies of this clade in the east Pearl River sub-region probably indicate that it is an ancestral clade (Crandall & Templeton, 1993). At least two major migratory routes can be inferred from the mtDNA gene genealogy: one from the east Pearl River sub-region (clade 2-2) through the Jiulongjiang River (clade 1-8) to the Hanjiang River (clade 1-9) and the other from the east Pearl River sub-region through the northern ZheMin sub-region (clade 1-4) to the East China region and west Pearl River sub-region (clade 2-1). Geological evidence indicates that most rivers in the southeast coastal districts, including the Jiulongjiang, Hanjiang and Moyangjiang rivers, did not form until the Quaternary (Tchang *et al.*, 1990; Zheng, 2004). Demographic expansions into these river systems may have occurred only recently, and this is concordant with the molecular dating from mtDNA markers. Thus, populations in Pearl and Minjiang rivers may represent refugia and centres of genetic diversity. In addition, migrations from populations in the Minjiang River to Xijiang River may have augmented genetic diversities in Pearl River populations.

Geological evidence also indicates that transformed river trajectories, rivers and mountain ranges greatly affected the migration of many species in East and South China (Tchang *et al.*, 1990; Rainboth, 1991). This geographical history is reflected in the apportionment of genetic variation among populations (Xiao *et al.*, 2001; Perdices *et al.*, 2004). In the present study, small dispersion values (Dc), significant displacement (Dn) from the clade centre (Table IVa)

and non-overlapping distributions indicate dispersals from isolated populations in both regions. Long-distance colonizations occurred following the settlement of populations of *G. fokiensis*, as suggested by the large displacements of tip clades within the clade 2-1 and small dispersion values and displacements for interior clades within the clade 2-2 [Table IV(a, b)].

Genetic profiles of *G. fokiensis* indicate recent population expansions. Negative values of Tajima's D and Fu's F_S appeared in nearly all the samples and indicate excesses of rare haplotypes, as expected from mutation-drift disequilibria during a population expansion (Tajima, 1983, 1989*b*; Fu, 1997). A multimodal mismatch histogram and a star-like phylogeny are also concordant with recent population growth (Slatkin & Hudson, 1991; Chiang *et al.*, 2004). Thus, Quaternary changes on the river configurations may have contributed to population growth in *G. fokiensis*.

GENETIC POPULATION STRUCTURE AND PHYLOGEOGRAPHY OF *G. HAINANENSIS*

The genetic analysis of *G. hainanensis* populations revealed large divergences between populations in Leizhou Peninsula and Hainan Island. This genetic isolation was likely triggered by the isolation of the island from adjacent mainland. AMOVA indicates that not only the genetic variation resides between lineages but also the significant genetic structuring occurs among the populations ($F_{ST} = 0{\cdot}801$). Molecular dating also indicates that the Hainan Island population has been isolated sufficiently long (0·72 MY) to achieve reciprocal monophyly from mainland populations.

The NJ tree identified two lineages in *G. hainanensis* (Fig. 2) encompassing samples from Hainan Island (α) and the Leizhou Peninsula (β), respectively. Haplotypes in lineage β, in the sample from the Nanliujiang River, were more closely related to haplotypes in the Lianjiang River than to those in the Jianjiang River. The Nanliujiang and Lianjiang rivers (west Leizhou Peninsula) drain into the Gulf of Tonkin, but the Jianjiang River (east Leizhou Peninsula) drains into South China Sea. Nested clade analyses also revealed a large congruence between mtDNA lineages in populations on east Leizhou Peninsula (clade 1-C), west Leizhou Peninsula (clade 2-A) and Hainan Island (clade 1-D). Surprisingly, clade 1-C of east Leizhou Peninsula is phylogenetically closer to clade 1-D of Hainan Island than to clade 2-A of west Leizhou Peninsula, even though populations of clades 1-C and 2-A are geographically closer.

During Pleistocene glaciations, the intraspecific phylogeography and genetic diversity of the Hainan sub-region drainage were inevitably affected by changes in temperature (Yap, 2002) and drops in sea level (Chiang *et al.*, 2001). At last, glacial maximum sea level dropped about 120 m, exposing the present-day Gulf of Tonkin (Morton & Blackmore, 2001). Hainan Island became a part of the coastal plain of the Asian continent. Rivers on east Leizhou Peninsula and east Hainan Island merged and ran into the South China Sea, while the rivers on west Leizhou Peninsula drained into the Gulf of Tonkin (Morton & Blackmore, 2001; Attwood *et al.*, 2004). At these times, migrants probably moved between east Leizhou Peninsula and east Hainan Island populations during floods at low elevations. Gene flow between these populations was interrupted when

Hainan Island was separated from the mainland by the sinking of QiongZhou Strait about 0·72 MYA (Morton & Blackmore, 2001; Yap, 2002). Nested clade analysis of *G. hainanensis* indicated allopatric fragmentation at both the one- and two-step clade levels [Table V(a, b)].

Significant negative values of F_S were observed in samples of *G. hainanensis* and may reflect a recent population expansion. The mismatch distributions of haplotypes in this taxon also indicated a recent population expansion. These results are largely consistent with phylogeographical inferences. Accordingly, climate changes and glaciations may have contributed to such population growth in *G. hainanensis*.

In summary, the deep mtDNA separations between haplotypes in the putative sub-species *G. f. fokiensis* and *G. f. hainanensis* indicate that these taxa have been isolated from one another for a considerable amount of time and deserve distinct species rank. Both tectonic activity over the past few million years and climate changes during the Pleistocene Ice Ages produced barriers and migration pathways that are reflected in the genetic structures of present-day populations.

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