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Phylogeography of *Sylvirana latouchii* (Anura, Ranidae) in Taiwan

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Biogeographic studies are important for understanding the natural history of faunas. To comprehend the geographical patterns of genetic variation in anurans in Taiwan, we investigated the genetic structure of *Sylvirana latouchii* (Anura, Ranidae) from 31 populations by using mitochondrial DNA (mtDNA) cytochrome *b* sequences. A neighbor-joining tree of 38 haplotypes revealed three major divergences in Taiwanese *S. latouchii*: the northern, western, and eastern-and-southern clades. Each clade was restricted to a single geographical district and showed obvious differentiation. The patterns of geographical divergence in this species reflect common historical events experienced by other native animals distributed in Taiwan. The order of divergence times between clades was inferred using a molecular clock test. The population relationship of *S. latouchii* between Taiwan and mainland China is discussed. Further study employing more populations of *S. latouchii* from mainland China is necessary to clarify the original geographical patterns and migratory history of this species.

Key words: *Sylvirana latouchii*, Taiwan, phylogeography, mtDNA, cytochrome *b*

INTRODUCTION

Molecular data have been widely applied in biogeographic studies in recent years. In general, animal populations are subject to being genetically structured by isolating factors such as oceans, rivers, and topographical barriers (Manel *et al*., 2003; Funk *et al*., 2005). Both historical events experienced by a species and its ecological characteristics influence the present genetic structure, and phylogeographic research using DNA markers provides valuable information concerning the historical factors (Hashiguchi *et al*., 2006).

The extents and patterns of animal diversification vary among species (Avise, 2000). In general, aquatic animals in terrestrial areas, such as freshwater fishes, provide excellent opportunities for historical biogeographic research because of their limited dispersal capabilities. Most amphibians have two phases in their life history. During their larval phase, amphibians usually confine their movements within aquatic environments. However, they are able to migrate across land during their terrestrial adult phase. The moist skin of amphibians is sensitive to humidity, which is affected by various conditions such as vegetation, light, temperature, rainfall and water availability in micro-environments; this makes them good indicators of environmental changes.

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Taiwan, a subtropical to tropical island located off the coast of southeastern China, has high biodiversity (Lee, 2004; Shao *et al.*, 2006). Uplift of the mountains caused by collision of the Eurasian and Philippine plates caused this island's steep topography about five million years ago (Ma) (Teng, 1990; Huang *et al*., 1997) and created a craggy Central Mountain Range about 2.50–1.00 Ma (Lin, 1966; Huang *et al*., 1997). At present, the Central Mountain Range reaches 3,952 m above sea level (Jade Mountain) and has more than 100 peaks over 3,000 m high. The Central Mountain Range is considered to be a major isolating mechanism preventing genetic interaction on this island. Furthermore, the flora and fauna are presumed to have diverged significantly owing to complicated topographical, climatic, and ecological patterns. The high biodiversity of Taiwan provides an interesting example for phylogeographic studies. Several phylogenetic and phylogeographic investigations using molecular approaches have been conducted on small vertebrates, in particular freshwater fishes (Wang *et al*., 1999; Wang *et al*., 2004; Cheng *et al*., 2005; Ma *et al*., 2006; Watanabe *et al*., 2007). Although Yang *et al*. (1994) studied an endemic treefrog, few amphibian studies of this kind have been reported from Taiwan.

Sylvirana latouchii is a widespread frog in the area from Jiangsu to Guizhou in China and in Taiwan (Fei, 1999; Lue *et al*., 1999). In Taiwan, it is a common species, distributed from the lowlands up to elevations ca. 1,500 m above sea level (Lue *et al*., 1999). Generally, *S. latouchii* breeds all

year (Huang *et al*., 2004), usually in slow-moving water in ditches and small streams, and in static rain pools (Chou and Lin, 1997; Wu and Kam, 2005). *Sylvirana latouchii* is a dominant amphibian species in the lowlands of Taiwan.

In this study, we investigated the biogeographical patterns of Taiwanese *S. latouchii* populations through mtDNA data. We propose a scenario to explain the phylogeographic patterns observed and compare these patterns with migration patterns deduced from other native animals of Taiwan.

MATERIALS AND METHODS

Sample collection

A total of 198 specimens of adult *Sylvirana latouchii* were collected from 31 sites in Taiwan. Additional samples were collected from China, including five individuals from three localities in Zhejiang and Anhui Provinces. The collecting sites are shown in Fig. 1. Muscle or liver tissue samples were preserved in 99.5% ethanol for laboratory analyses. Most specimens used in this study were deposited in the National Museum of Natural Science (NMNS), Taichung, Taiwan (Table 1).

DNA amplification and sequencing

Genomic DNA was isolated from a piece of muscle or liver tissue (about 5 mg) using the Tissue and Cell Genomic DNA Purification Kit (Hopegen Biotechnology Development Enterprises). The extraction of crude DNA was performed according to the manufacturer's instructions with repeat membrane binding, salt washing, and centrifugation. A 638-bp region of the cytochrome *b* gene was selected for amplification with the polymerase chain reaction (PCR) using primers L14850 (5'-TCTCATCCTGATGAAACTTTGGCTC-3') and H15502 (5'-GGATTAGCTGGTGTGAAATTGTCTGGG-3') designed by Tanaka-Ueno *et al*. (1998). PCR conditions consisted 35 cycles of denaturation (95°C, 50 s), annealing (46°C, 1 min), and extension (72°C, 1min 20 s) (modified from Saiki, 1990) on a Mastercycler gradient 5531 PCR cycler (Eppendorf Inc.) with PCR Master Mix Kit (Hopegen Biotechnology Development Enterprises). PCR products were purified with the PCR-M Clean Up System kit (Viogene Inc.) and used for sequencing. Sequences were obtained by the multiple fluorescent dyes method using an Applied Biosystem 377 automatic sequencer, and were aligned with the aid of MegAlign ver. 4.0 (DNA Star Inc.) and by eye, using the complementary strand for verification. Sequences from 203 specimens, including those from Taiwan and China, were deposited

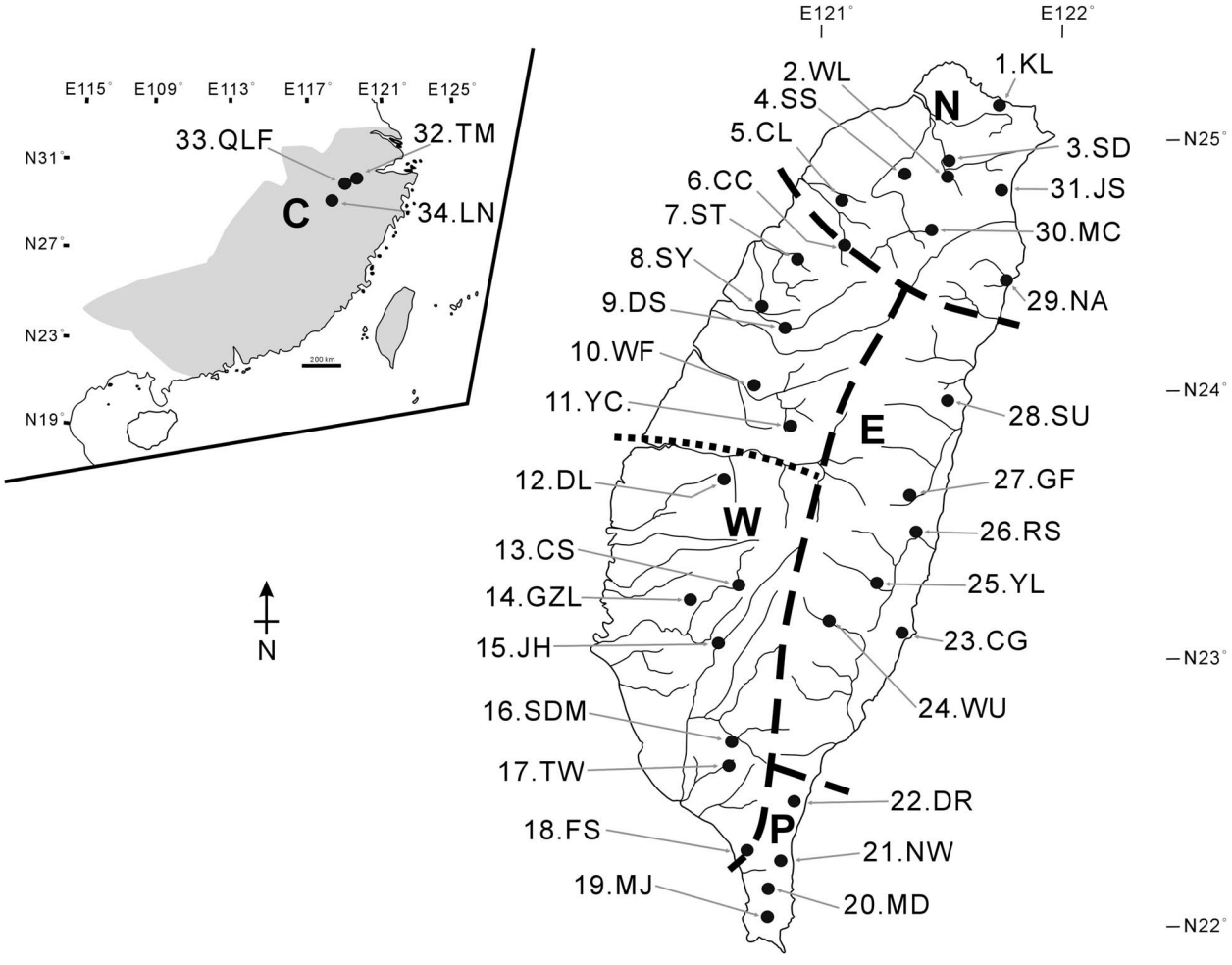

Fig. 1. Locations of sampling sites for *Sylvirana latouchii*. Site numbers and locality codes are listed in Table 1. Dashed lines represent postulated boundaries of four biogeographic districts in Taiwan based on the results of phylogenetic analyses: Northern District (N), Western District (W), Eastern District (E) and Hengchun Peninsula District (P). The dotted line represents a possible geographic isolating feature, the Chousui River, within the Western District. The gray-shaded zone (Mainland China District; C) denotes the distributional range of *S. latouchii* (Fei, 1999).

Table 1. Sampling localities, sample sizes (Ns), haplotypes and specimen numbers analyzed for *Sylvirana latouchii.*

Locality (code)	Ns	Haplotypes (no. of individuals)	Specimen (NMNS*)
1. Keelung (KL)	2	h1(1), h2(1)	15599, **
2. Wulai (WL)	9	h2(4), h3(4), h4(1)	10630–10633, 10635, 16413–16416
3. Sindian (SD)	4	h2(3), h5(1)	16408–16411
4. Sansai (SS)	5	h2(2), h6(1), h7(2)	16150–16154
5. Cyonglin (CL)	10	h2(8), h8(2)	16170–16179
6. Chinchun (CC)	5	h2(1), h6(3), h9(1)	16447–16451
7. Shihtan (ST)	6	h9(2), h10(4)	16389–16394
8. Sanyi (SY)	10	h9(7), h11(3)	15529–15538
9. DongShih (DS)	2	h9(1), h12(1)	16470–16471
10. Wufong (WF)	7	h9(1), h11(4), h13(1), h14(1)	15545–15551
11. Yuchih (YC)	7	h9(5), h15(1), h16(1)	15865–15866, 16372–16376
12. Douliou (DL)	10	h11(4), h17(1), 18(2), 19(1), 20(1), h21(1)	15488–15490, 16018–16024
13. Chiashan (CS)	7	h16(3), h21(1), 22(1), 23(1), 24(1)	16399–16405
14. Guanziling (GZL)	10	h16(2), h21(2), 23(4), 25(2)	10677–10679, 15723–15728, 15731
15. Jiashian (JH)	10	h16(5), h21(5)	16087–16089, 16096–16097, 16101–16105
16. Sandimen (SDM)	10	h23(10)	16131–16140
17. Taiwu (TW)	6	h21(2), h23(2), 26(1), 27(1)	16464–16469
18. Fangshan (FS)	5	h28(4), h29(1)	15693–15697
19. Manjhou (MJ)	9	h28(8), h30(1)	15090–15092, 15455–15456, 16140–16143
20. Mudan (MD)	2	h28(2)	15434–15435
21. Neiwen (NW)	3	h28(3)	16133–16134, 16136
22. Daren (DR)	10	h28(10)	16110–16119
23. Chenggong (CG)	8	h31(7), h32(1)	16326–16330, 16396–16398
24. Wulu (WU)	5	h31(5)	16438–16442
25. Yuli (YL)	3	h31(2), h33(1)	15660–15662
26. Rueisuei (RS)	7	h31(5), h34(2)	15977–15978, 15986–15989, 15996
27. Guanfu (GF)	4	h31(3), h35(1)	15828–15830, 16395
28. Sueiyuan (SU)	6	h35(5), h36(1)	16429–16434
29. Nan-oa (NA)	10	h3(2), h37(8)	15961–15970
30. Mingchr (MC)	2	h2(1), h37(1)	16443–16444
31. Jiaosi (JS)	4	h3(2), h37(1), 38(1)	15871–15872, 16332, **
32. Tianmu Mt. (TM)	2	h39(1), h40(1)	16214, 16240
33. Qingliangfeng (QLF)	1	h41(1)	16261
34. Linnan (LN)	2	h42(1), h43(1)	16294, **
Total	203		

* Acronym of the National Museum of Natural Science, Taichung, Taiwan.

** uncatalogued specimens.

in the GenBank database (accession numbers EU034727–EU034929).

Data analysis

Preliminary phylogenetic and molecular evolutionary analyses were conducted using MEGA version 3.1 (Kumar *et al*., 2004) and DNA SP version 4.0 (Rozas *et al*., 2003). We constructed phylogenetic trees using neighbor-joining (NJ), maximum parsimony (MP), and maximum likelihood (ML) analyses. All these analyses were performed on unique haplotypes by PAUP* version 4 beta (Swofford, 2001). Likelihood settings from the best-fit model (HKY+G) (gamma correction=0.1705) with base frequencies of A=0.2491, C=0.3019, G=0.1572, T=0.2918 and a transition/transversion ratio of 14.5122, selected by hLRTs (hierarchical likelihood ratio tests) were obtained from MODELTEST 3.7 (Posada and Crandall, 1998). Replicate haplotypes were excluded from the analysis to reduce computational time. NJ tree construction was based on the probability model identified above, with ties broken randomly. MP and ML analyses were conducted using a random addition heuristic search with tree-bisection-reconnection (TBR) branch swapping. Bootstrapping [1,000 replicates for NJ (NJ option), MP (fast-heuristic search), and ML (full-heuristic search, TBR branch swapping)] was performed to obtain a relative measure of node support for the resulting tree (Felsenstein, 1985). The selective neutrality of all sequences was assessed by Tajima's *D* (Tajima, 1989) statistic and Fu and Li's *D* and *F* statistics (Fu and Li, 1993) within each clade and local population.

A nested clade network was constructed by linking haplotypes in a hierarchical manner based on the variations between sequences. For the purpose of analyzing the possibilities of genetic relationships between populations, nested clade analysis is useful and expected. In this study, we used two programs to reconstruct the parsimonious network for all haplotypes of *S. latouchii* populations. MINSPNET (Excoffier and Smouse, 1994) was applied for an overview of a network and TCS 1.21 (Clement *et al*., 2000) was used for detailed networks of intra-lineage relationships.

For estimating divergence times between clades, we conducted the two-cluster test of constancy of evolutionary rates based on the K2P-distance model for all haplotypes using LINTREE (Takezaki *et al*., 1995). The test employed the NJ method to establish the tree topology, and *Rana sauteri* (GenBank accession number EU034930) was used as the outgroup. We calculated the height of the branch point of two clades, defined as one-half the average of the mean nucleotide differences between the two clades. Diver-

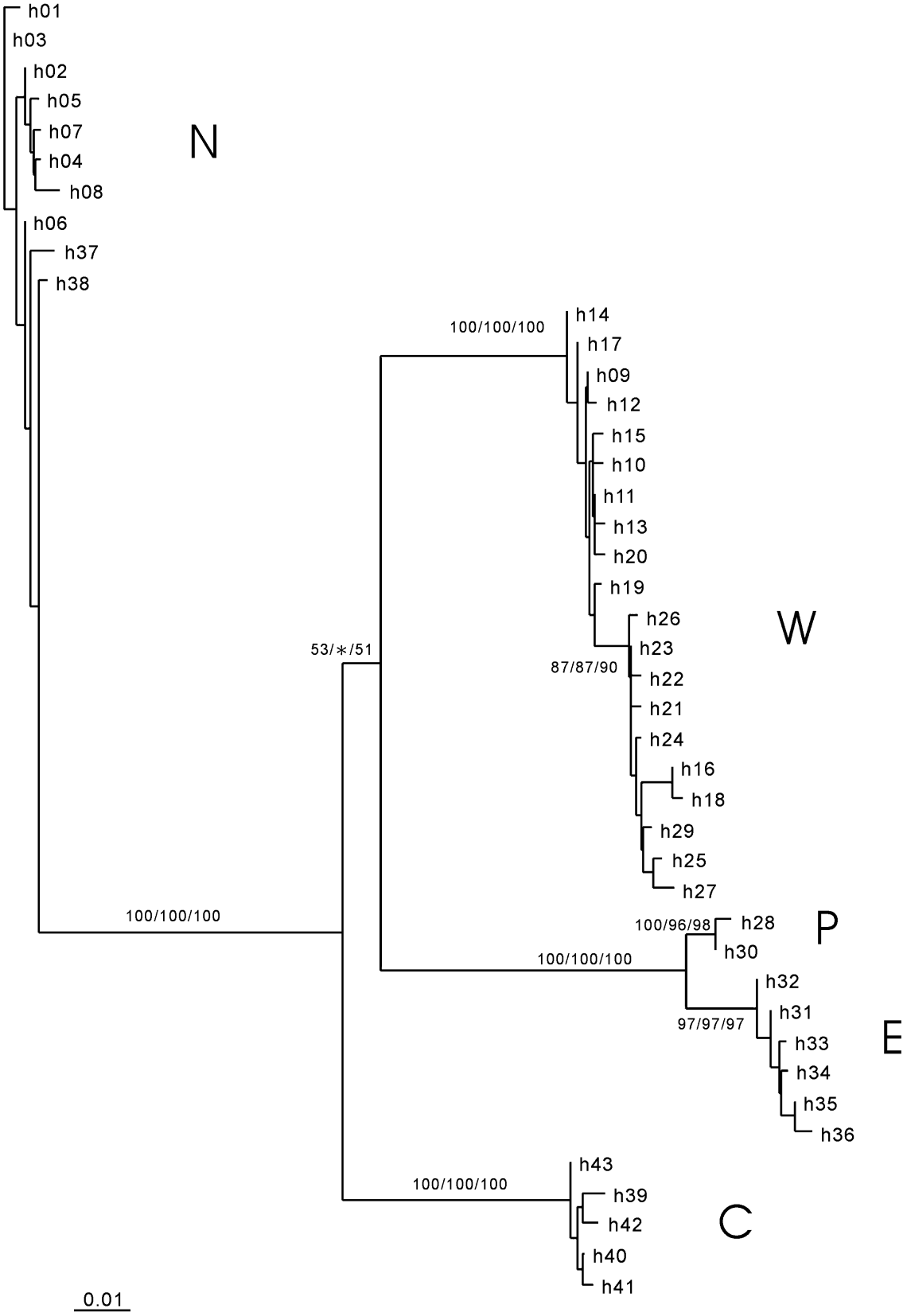

Fig. 2. Neighbor-joining tree of 638-bp-long haplotypes inferred from partial cytochrome b sequences. Branch lengths are proportional to the scale given in nucleotide substitutions per site. Numbers at internal nodes are bootstrap values (>50%) for the NJ, MP and ML analyses. Only values for major clades or sub-clades are shown. Abbreviations beside clades or sub-clades (N, Northern clade; W, Western clade; P, Hengchun Peninsula sub-clade; E, Eastern sub-clade; C, mainland China clade) correspond to the biogeographical districts defined in Fig. 1.

gence time between clades was estimated by the ratio of the height to the divergence rate. In this study, we applied 1.41% /million years (Myr) as the divergence rate deduced from the divergence event of clade E from other populations (the height is 0.0353; see Table 6), and the geological event of the Central Mountain Range formation, about 2.50–1.00 Ma (Lin, 1966; Huang *et al*., 1997). We used 2.50 Ma as the beginning of divergence time for estimating the divergence rate, presuming that the Central Mountain Range had reached a

Table 2. *F*st value inferred from all sequences (above diagonal) and estimated pairwise distant value inferred from haplotypes of *Sylvirana latouchii* partial cytochrome *b* sequence by K2P model (below diagonal) between major clades or sub-clades (see Fig. 2 for abbreviations). Distance values within clades/sub-clades inferred from haplotypes are also shown beside the data matrix.

	1	2	3	4	5	6	d×10^2 within clade/sub-clade
1. N		0.9213	0.9734	0.9684	0.9129	0.9361	0.4552
2. W	0.0682		0.9459	0.9420	0.8818	0.9075	0.8217
3. P	0.0707	0.0649		0.9657	–	0.9689	0.1570
4. E	0.0760	0.0702	0.0167		–	0.9607	0.2936
5. E+P	0.0745	0.0689	–	–		0.9047	0.8801
6. C	0.0625	0.0607	0.0712	0.0697	0.0700		0.4095

Distance overall value inferred from haplotypes=0.0495±0.0053.

Table 3. Genetic variation of sampling localities of *Sylvirana latouchii* in this study. See Table 1 for locality codes and Fig. 2 for abbreviations of clades/sub-clades.

			Numbers of individuals of each clade/sub-clade								
Locality code	Ns	*h*	**N**	**W**	***P***	***E***	**E+P**	**C**	*s*	Hd	π×10^2
1. KL	2	2	2	–	–	–	–	–	3	1.000	0.470
2. WL	9	3	9	–	–	–	–	–	2	0.667	0.209
3. SD	4	2	4	–	–	–	–	–	3	0.500	0.078
4. SS	5	3	5	–	–	–	–	–	1	0.800	0.157
5. CL	10	2	10	–	–	–	–	–	2	0.356	0.111
6. CC	5	3	4	1	–	–	–	–	38	0.700	2.382
7. ST	6	2	–	6	–	–	–	–	2	0.533	0.167
8. SY	10	2	–	10	–	–	–	–	1	0.467	0.073
9. DS	2	2	–	2	–	–	–	–	1	1.000	0.157
10. WF	7	4	–	7	–	–	–	–	3	0.714	0.164
11. YC	7	3	–	7	–	–	–	–	9	0.524	0.463
12. DL	10	6	–	10	–	–	–	–	13	0.844	0.731
13. CS	7	5	–	7	–	–	–	–	6	0.857	0.448
14. GZL	10	4	–	10	–	–	–	–	7	0.800	0.418
15. JH	10	2	–	10	–	–	–	–	5	0.556	0.435
16. SDM	10	1	–	10	–	–	–	–	0	0.000	0.000
17. TW	6	4	–	6	–	–	–	–	6	0.867	0.345
18. FS	5	2	–	1	*4*	–	4	–	42	0.400	2.633
19. MJ	9	2	–	–	*9*	–	9	–	1	0.222	0.035
20. MD	2	1	–	–	*2*	–	2	–	0	0.000	0.000
21. NW	3	1	–	–	*3*	–	3	–	0	0.000	0.000
22. DR	10	1	–	–	*10*	–	10	–	0	0.000	0.000
23. CG	8	2	–	–	–	*8*	8	–	1	0.250	0.039
24. WU	5	1	–	–	–	*5*	5	–	0	0.000	0.000
25. YL	3	2	–	–	–	*3*	3	–	1	0.667	0.104
26. RS	7	2	–	–	–	*7*	7	–	1	0.476	0.075
27. GF	4	2	–	–	–	*4*	4	–	1	0.500	0.078
28. SU	6	2	–	–	–	*6*	6	–	1	0.333	0.052
29. NA	10	2	10	–	–	–	–	–	4	0.356	0.223
30. MC	2	2	2	–	–	–	–	–	4	1.000	0.627
31. JS	4	3	4	–	–	–	–	–	6	0.833	0.496
32. TM	2	2	–	–	–	–	–	2	3	1.000	0.470
33. QLF	1	1	–	–	–	–	–	1	n/c	n/c	n/c
34. LN	2	2	–	–	–	–	–	2	2	1.000	0.313
All localities	203	43	50	87	*28*	*33*	61	5	103	0.936	4.520

Ns, number of specimens; *h*, number of haplotypes observed; *s*, number of segregating sites; Hd, estimates of haplotype diversity; π, nucleotide diversity.

height sufficient to separate the populations on either side of it.

RESULTS

Phylogenetic analysis

In this study, partial cytochrome *b* gene sequences were amplified from 198 Taiwanese *Sylvirana latouchii* specimens and five individuals from China. The sequences are all 638 bp long, without insertions, deletions, or stop codons. In total, 103 polymorphic sites were identified, and among them 82 sites were parsimony informative.

A total of 43 haplotypes were detected from all specimens. The neighbor-joining haplotype tree with NJ, MP and ML bootstrap values (Fig. 2) shows four major clades: 1) clade N, defined by 10 haplotypes from 50 specimens representing northern populations in Taiwan; 2) clade C, defined by specimens from mainland China; 3) clade W, defined by 20 haplotypes from 87 specimens representing western populations in Taiwan; and 4) clade E+P, defined by eight haplotypes from 62 specimens from the eastern part (sub-clade E) and southern peninsula (sub-clade P) of Taiwan. Sub-clade E was composed of six haplotypes from 33 specimens collected from eastern part of the Central Mountain Range. The sub-clade P had two haplotypes identified from 28 specimens collected from the Hengchun Peninsula at the southern tip of Taiwan. The strict consensus trees from MP and ML analyses (not shown) exhibited no discrepancies with the NJ tree in the topology of the major clades. Fig. 1 shows the biogeographic distributions of these clades and sub-clades.

The average sequence difference among all haplotypes was 4.95±0.53% (mean±SD; range, 0.16–8.30%). There were 38 haplotypes in Taiwanese *S. latouchii* specimens. Among Taiwanese specimens, the average sequence difference was 4.62±0.53% (range 0.16–8.30%). Among specimens from China, the average sequence difference was 0.41±0.16% (range 0.16–0.63%). *F*st values were high (0.88–0.97) between major clades inferred from all sequences. Distances between clades inferred from haplotypes ranged from 1.67–7.60%, and those within clades, from 0.16–0.88% (Table 2).

Within-population genetic variation (nucleotide diversity, π) among 33 local populations, excluding Qingliangfeng (site 33) of China where only one specimen was collected, ranged from 0.00–2.63%; the mean of all individuals was 4.52% (SD±0.08%). Haplotype diversity (Hd) within each locality ranged from 0.00–1.00 (Table 3). Nucleotide diversity (π) among clades ranged from 0.01–0.85%. Haplotype diversity (Hd) within each clade ranged from 0.07–0.88 (except for 1.00 for the Chinese clade) (Table 4).

Table 4. Genetic variation of clades/sub-clades of *Sylvirana latouchii* in this study. See Fig. 2 for abbreviations of clades/sub-clades.

	Clade or sub-clade					
	N	**W**	***P***	***E***	**E+P**	**C**
Ns	50	87	28	33	61	5
h	10	20	2	6	8	5
Hd	0.779	0.881	0.071	0.532	0.673	1.000
$\pi\times10^2$	0.342	0.657	0.011	0.101	0.849	0.408
$d\times10^2$	0.344	0.663	0.011	0.101	0.862	0.410

Ns, number of specimens; *h*, number of haplotypes observed; Hd, estimates of haplotype diversity; π, nucleotide diversity; d, distance by K2P model inferred from all individuals.

Neutrality tests

Neutral theory has become the primary null hypothesis used to test for the effects of natural selection. If a molecular marker that is assumed to be evolving neutrally is actually subject to selection, conclusions based on patterns of dis-

Table 5. Neutrality test statistics of *Sylvirana latouchii* within each population (clades/sub-clades and localities; Figs. 1 and 2) using the total number of mutations of partial cytochrome *b* sequence for calculations.

Clade-Locality	n	Tajima's *D*	Fu and Li's *D*	Fu and Li's *F*
Clade N	50	–0.5478	–0.6843	–0.7543
1. KL	2	n/c	n/c	n/c
2. WL	9	0.7944	0.2314	0.4045
3. SD	4	–0.6124	–0.6124	–0.4787
4. SS	5	0.2431	0.2431	0.2386
5. CL	10	0.0189	1.0262	0.8732
6. CC	5	–1.2522	–1.2522	–1.3530
29. NA	10	0.0225	1.2391	1.0548
30. MC	2	n/c	n/c	n/c
31. JS	4	–0.3145	–0.3145	–0.3023
Clade W	87	–0.2465	–1.3171	–1.0940
7. ST	6	1.0319	1.2797	1.2748
8. SY	10	0.8198	0.8042	0.8978
9. DS	2	n/c	n/c	n/c
10. WF	7	–0.6541	–0.5190	–0.5921
11. YC	7	–1.0433	–0.9711	–1.0811
12. DL	10	0.0707	0.6184	0.5434
13. CS	7	0.8467	0.4388	0.5775
14. GZL	10	0.3291	1.3822*	1.2610
15. JH	10	2.2922*	1.3001	1.7294**
16. SDM	10	–	–	–
17. TW	6	–0.9317	–0.9082	–0.9766
18. FS	5	–1.2454	–1.2544	–1.3566
Clade E+P	61	2.0484	0.1253	0.9402
Sub-clade P	28	–1.1514	–1.6591	–1.7474
19. MJ	9	–1.0882	–1.1899	–1.2829
20. MD	2	n/c	n/c	n/c
21. NW	3	n/c	n/c	n/c
22. DR	10	–	–	–
Sub-clade E	33	–1.2713	–1.5448	–1.7036
23. CG	8	–1.0548	–1.1264	–1.2035
24. WU	5	–	–	–
25. YL	3	n/c	n/c	n/c
26. RS	7	0.5590	0.9535	0.9179
27. GF	4	–0.6124	–0.6124	–0.4787
28. SU	6	–0.9330	–0.9502	–0.9647
Clade C	5	–0.6682	–0.6682	–0.6924
32. TM	2	n/c	n/c	n/c
33. QLF	1	n/c	n/c	n/c
34. LN	2	n/c	n/c	n/c
All specimens	203	2.0078	0.8621	1.6741

n: numbers of sequences.
*: statistically significant at the 5% level.
**: statistically significant at the 2% level.
n/c: not calculated because of too small number of sequences (less than 4 sequences).
–: not calculated due to lack of polymorphisms in data.

similarity at the marker could be misleading (Ford, 2002). The values of neutrality test statistics are shown in Table 5. Tajima's *D* values did not significantly deviate from zero in most localities, except for the Jiashian population (site 15). Fu and Li's *D* and *F* values did not deviate significantly from zero in most populations, except for the Guanziling (site 14) and Jiashian (site 15) populations, respectively. No neutrality test values among clades N, W, E+P, and C significantly deviated from zero. These results indicate that the cytochrome *b* gene of *S. latouchii* evolves neutrally, and can be considered as a neutral marker.

Nested clade analysis and phylogeographic information

A minimum spanning network was constructed by linking the haplotypes in a hierarchical manner based on variation between sequences (Fig. 3). In this network, four major clades were widely separated and largely agreed with the neighbor-joining tree. Few haplotypes were located at the interior nodes between clades. Detailed haplotype networks of each major clade constructed by TCS 1.21 are shown in Fig. 4. In clade N that comprised samples from nine collecting sites, one major haplotype (h02, represented by 20 individuals) was distributed in seven localities. Only two eastern coast localities, Nan-oa (site 29) and Jiaosi (site 31), lacked h02 in the specimens sampled. Haplotypes h03 and h37 from eight and 10 individuals, respectively, were both distributed in three localities. Other haplotypes were distributed in one or two localities with no more than four individuals. In clade W, there were 20 haplotypes, representing 87 individuals from 13 localities. Haplotypes h09, h11, h16, h21, and h23, each represented by more than 10 individuals, constituted phylogeographic centers in the district of clade W. Chinchun (site 6), having both N haplotypes (h2 and h6) and a W haplotype (h9) and considered a boundary locality between clades N and W, had a high π value (π=2.38%). This high π value resulted from a remarkably large number of segregating sites (s=38) among the populations sampled (Table 4). Likewise, Fangshan (site 18), with haplotypes from both clade W and sub-clade P, had a high π value (π=2.63%) and many segregating sites (s=42), and was considered a boundary locality. In clade E+P, sub-clades P and E appeared to be sister groups. The haplotype diversity (Hd) of clade E+P is 0.673; the Hds of sub-clades P and E are 0.071 and 0.532, respectively. The two sub-clades included haplotypes h28 and h31, respectively, which were distributed in all sampling localities of both districts. Among 28 individuals of sub-clade P, haplotype h28 was found in 27 individuals; the other haplotype, h30, with a single mutational difference from h28, occurred in the other individual. In sub-clade E, haplotype h31 occurred in 22 individuals

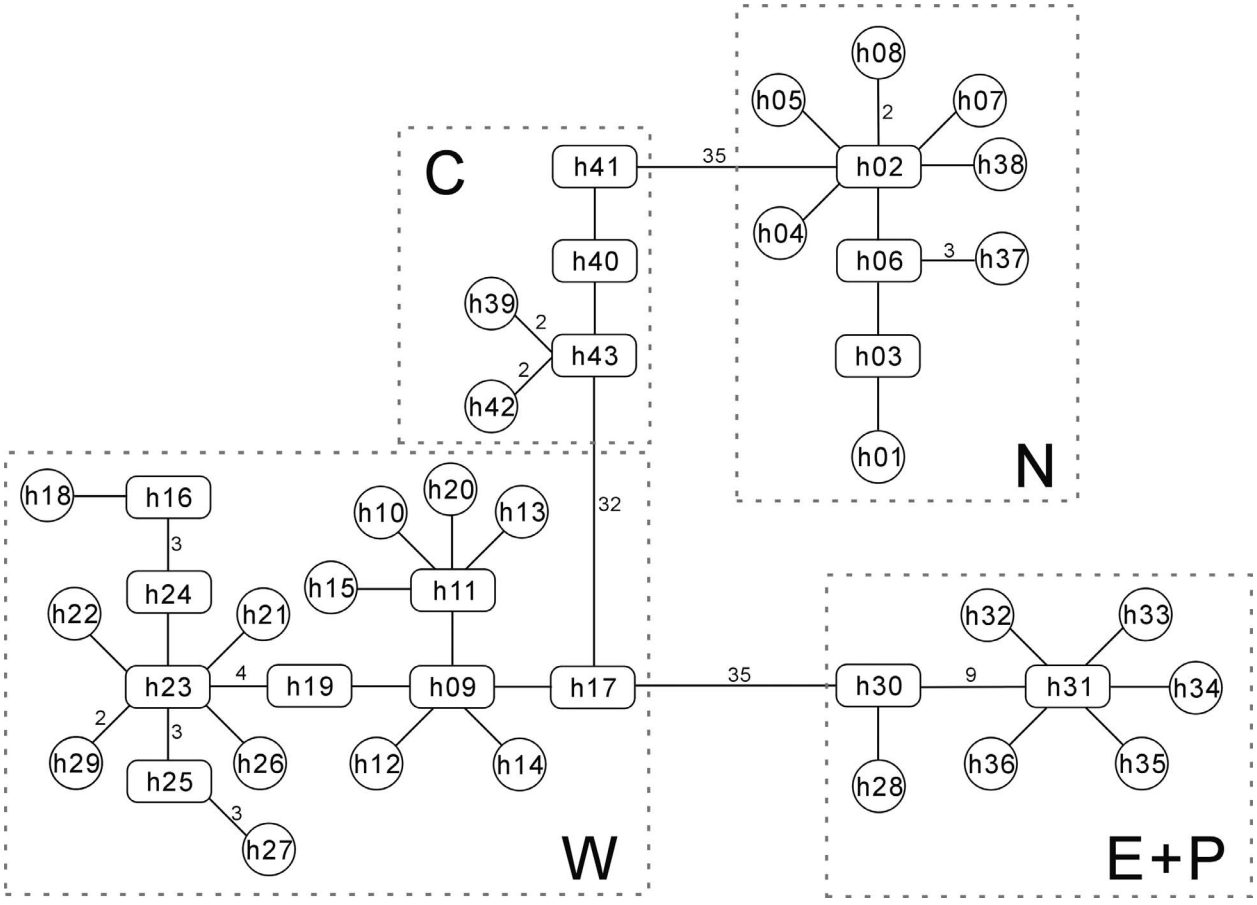

Fig. 3. Minimum spanning network based on variation between haplotype sequences of populations of *Sylvirana latouchii*. Numbers at nodes indicate the number of nucleotide changes between haplotypes. Nodes without a number represent a single nucleotide change between haplotypes.

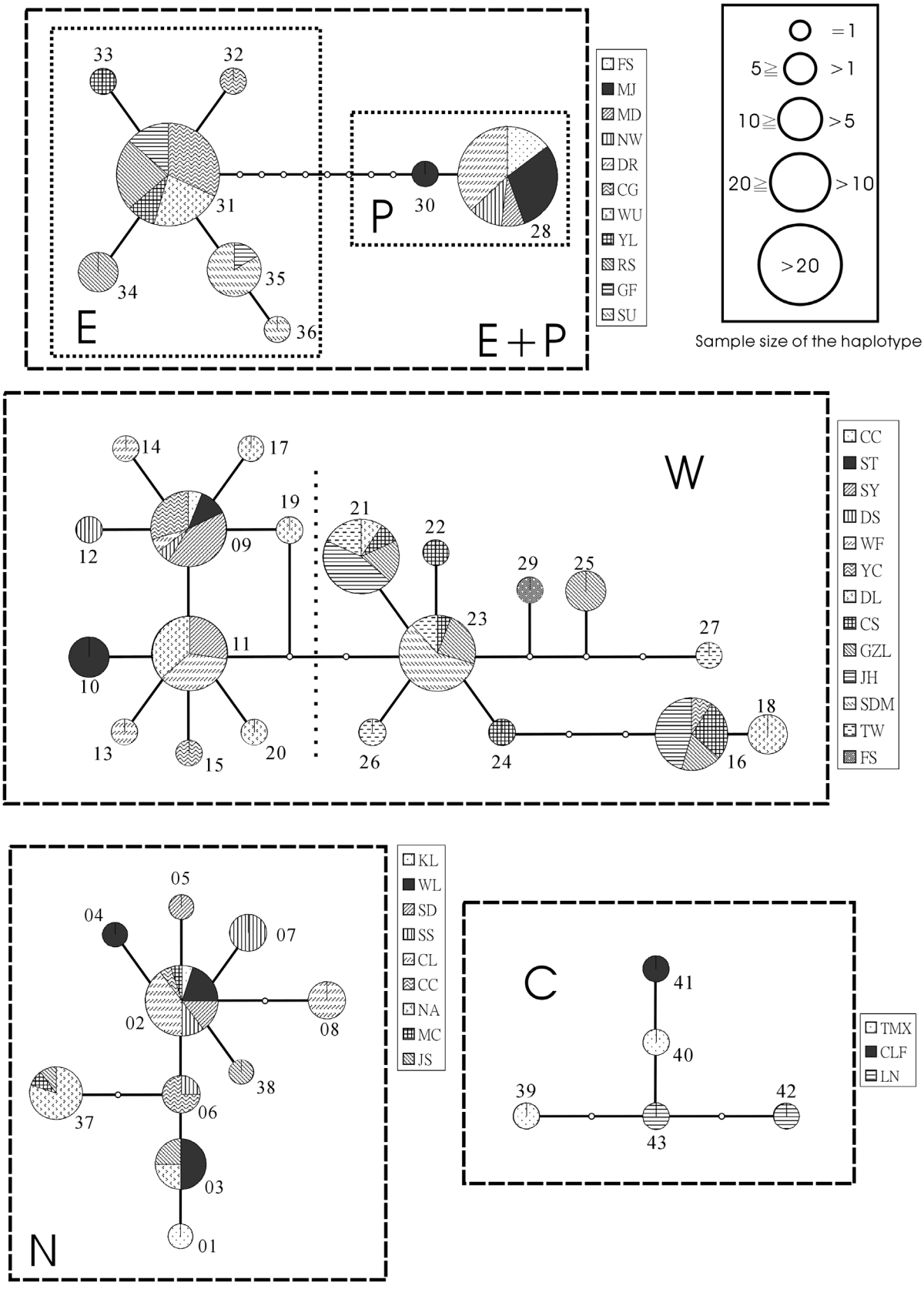

Fig. 4. Detailed parsimony network for each major clade, constructed by TCS 1.21. Numbers beside pie-charts are haplotypes. Circle size reflects the number of individuals having the corresponding haplotype. Small open circles signify possible missing haplotypes. A line connecting two haplotypes represents one nucleotide substitution. See Table 1 for sampling locality codes.

Table 6. Results of two-cluster test and estimated divergence times of *Sylvirana latouchii* inferred from haplotype clades/sub-clades (Fig. 2). Divergence time was calculated from its height.

Sister groups (1 vs. 2)		CP(%)	Z	delta	SE	b_1	b_2	height (SE)	divergence time (mean±SE; Ma)
N	vs. C	53.46%	0.7348	0.0067	0.0091	0.0346	0.0279	0.0313 (0.0051)	2.22±0.36
(N+C)	vs. W	46.48%	0.6287	0.0053	0.0085	0.0355	0.0302	0.0328 (0.0045)	2.32±0.32
P	vs. E	66.80%	0.9782	0.0045	0.0046	0.0061	0.0106	0.0084 (0.0025)	0.59±0.18
E+P	vs. (W+N+C)	73.30%	1.1198	0.0113	0.0101	0.0297	0.0410	0.0353 (0.0045)	2.50

Z=delta/SE; delta=| b_1–b_2 |; height=average (b_1, b_2); divergence time=height/divergence rate (=1.41% Myr in this study); and CP value=1–p value.

among 33 specimens. Altogether, there were eight missing haplotypes distributed between sub-clades P and E (Fig. 4, E+P).

Divergence times among major clades within *Sylvirana latouchii*

We conducted the two-cluster test by including all 43 haplotypes used for the phylogenetic analysis, with a cytochrome *b* sequence from *Rana sauteri* as the outgroup. The results of the test are summarized in Table 6. The CP value is the confidence probability (1–*P*-value) computed in the two-cluster test. Differentiation events between clades/sub-clades showed no significant rate differences. The divergence time between major clades within Taiwan was estimated to be 2.22 (±0.36 SE) to 2.50 million years ago (Ma). The divergence time between the Taiwanese and Chinese populations (clade N vs. clade C) was estimated to be 2.22±0.36 (mean±SE) Ma. Within Taiwanese sub-clades, the estimated divergence time between sub-clades P and E was 0.59±0.18 Ma. These results suggest that divergences of the major clades occurred in the late Pliocene.

DISCUSSION

Genetic structure and population history of *Sylvirana latouchii*

Our phylogenetic analyses revealed four major clades in *Sylvirana latouchii* (Fig. 2). Divergences between the major clades [i.e., clade N vs. clade C, clades (N+C) vs. clade W, and clade E+P vs. clades (W+N+C)] seem to have occurred at almost the same time (2.50–2.22 Ma) (Table 6). The boundaries between clades inferred from the genetic data are distinct. We did not find continuous genetic changes among populations between major clades. There are two possible reasons to explain the discontinuous dispersal: efficienct topographic barriers and different migration events.

The Taiwan Strait and the Central Mountain Range seem to be efficient isolating mechanisms to disrupt gene flow within *S. latouchii* populations, as shown by the distinct phylogenetic clades. Inside Taiwan, the Central Mountain Range, running from the north to the south of the island, is the main topographic barrier that disrupts gene flow between eastern and western Taiwanese clades. The rise of the Central Mountain Range occurred about 2.50–1.00 Ma (Lin, 1966; Huang *et al*., 1997). We assume that during this period, the Central Mountain Range reached a height sufficient to separate populations of *S. latouchii* on either of its two sides. Based on contemporary understanding of this geological event, the estimated divergence rate of *S. latouchii* clades on the two sides of the Central Mountain Range is 1.41% Myr. This divergence rate is similar to the mtDNA divergence rates (%/Myr) for hynobiid salamanders (1.28%; Weisrock *et al.*, 2001; Tominaga *et al.*, 2006), toads (1.38%; Macey *et al.*,1998), pelobatid frogs (1.7%; Veith *et al.*, 2006), and vertebrates (2%; Brown *et al*., 1979).

The Taiwan Strait, about 200 km wide and 50 m deep on average, separates Taiwan from the Chinese mainland and has interrupted gene flow between organisms distributed on either side of it. Animal fossil records, including elephants and deer, indicate that Taiwan was connected to mainland China before the Pleistocene (Shikama *et al*., 1975; Otsuka and Shikama, 1978; Otsuka, 1984; Qi *et al*., 1999). From geological evidence, it is widely accepted that Taiwan has been connected with and then subsequently isolated from the Chinese continent more than once because of the interaction of arc-continent collisions and sea level changes during the Pleistocene Ice Ages (Emery *et al*., 1971; Zhao, 1982; Yang, 1991). Periodic episodes of glaciation during the Pleistocene resulted in numerous speciation events within the Taiwanese fauna and flora. Glaciations might also have provided opportunities for *S. latouchii* to migrate between mainland China and Taiwan. Our data suggest there could be two divergence events related to isolation by the Taiwan Strait. The first is divergence between the western Taiwan (clade W) and Chinese populations at about 2.32±0.32 Ma, according to the time of appearance of the Taiwan Strait. The second divergence event probably occurred on mainland China about 2.22±0.36 Ma, at which time the ancestors of clade N arose and crossed westward to Taiwan Strait via a land bridge during a later glacial period.

The distribution of clade N is limited to northern Taiwan, with Chinchun (site 6) and Nan-oa (site 29) as boundary localities on the western and eastern sides of the Central Mountain Range, respectively. On the eastern side, Chingshui Cliff, 21 km long and located between Sueiyuan (site 28) and Nan-oa (site 29), is an effective, hard-to-overcome geologic boundary that interrupts gene flow between northern and southern freshwater animals. On the western site, the boundary across Chinchun separating clades N and W lies on the northern side of the Miaoli Plateau. The Miaoli Plateau was estimated to have formed 0.15 Ma (see Tzeng, 1986), so it could therefore not be an isolation mechanism responsible for this earlier divergence event (2.32±0.32 Ma). We invoke a secondary invasion hypothesis to provide a possible explanation for the differentiation of clade N, which was originally from the Chinese continent and had probably diverged before migration to Taiwan. The multiple-invasion hypothesis regarding populations of the Taiwanese fauna

has been suggested by studies on several animals, including an endemic minnow (Wang *et al*., 1999), the bamboo viper (Creer *et al*., 2001; Creer *et al*., 2004), the white-bellied rat (Yu, 1995; Yu *et al*., 1996; Hsu *et al*., 2000), and Pallas's squirrel (Oshida *et al*., 2006). The mechanism that separated clades C and N is currently unknown, due to a lack of information pertaining to the phylogeography of *S. latouchii* on mainland China. In order to test the secondary invasion hypothesis for *S. latouchii* in Taiwan, more molecular data from mainland Chinese populations, especially from nearby Fujian Province, will be needed. A similar divergence event in *S. latouchii* should have occurred on the Chinese continent.

Sub-clades P and E were estimated to have diverged 0.59±0.18 Ma (Table 6). A possible boundary that divided sub-clades E and P is located between Daren (site 22) and Chenggong (site 23). This area is occupied by young mountains and cliffs, which interrupt the connection of the river systems between the Hengchun Peninsula and eastern Taiwan. Since this isolation event is still quite recent, it did not affect the continuity of distribution of freshwater fishes like *Varicorhinus alticorpus* and *Spinibarbus hollandi* (see Tzeng, 1986; Cheng and Fang, 1999), but it did become an isolating factor at the genetic level for freshwater fishes like *Varicorhinus barbatulus* (Wang *et al*., 2004). Our data indicate it could also be a barrier at the genetic level for amphibians. Furthermore, the only area occupied by sub-clade P, Hengchun Peninsula, is located at the southern extremity of Taiwan and belongs to the youngest part of the collision zone (Pelletier and Stephan, 1986). The model of geological evolution of this area is still uncertain. It is believed that the Hengchun Peninsula was built up about the time of the Pliocene- Pleistocene because of the arc-continent collision movement (Yu and Lu, 1995). Our estimated time of *S. latouchii* divergence suggests that the environmental situation for this species on the Hengchun Peninsula has been stablized since 0.59±0.18 Ma, owing to the peninsula effect.

The most recent divergence event within Taiwanese *S. latouchii* is a phylogenetic group represented within clade W. In this clade, 10 haplotypes (h16, h18, h21-27, h29) grouped together (Fig. 2). These haplotypes were distributed in the southern part of this clade from Yuchih (site 11) to Fangshan (site 18). The other ten haplotypes were distributed in northern part of clade W, from Chinchun (site 6) to Douliou (site 12). The boundary separating these two groups of haplotypes is between Yuchih and Douliou (dotted line in Fig. 1). These two localities are on opposite sides of the Chousui River, the longest river in Taiwan. It is possible that the Chousui River was wide enough to impede most lowland *S. latouchii* adult females from crossing. We suggest that clade W was isolated and diverged due to a topographic barrier, but that the divergence across the Chousui River was more recent, and migration events of a few individuals to and from both sides of Chousui River still occur. This recent divergence event apparently differentiates western-middle and western-southern populations of *S. latouchii* in Taiwan. Similar dispersal patterns have also been inferred from mtDNA data in phylogeographic studies of *Varicorhinus barbatulus* and Taiwanese bagrid catfishes (Wang *et al*., 2004; Watanabe *et al*., 2007).

The migration scenario of Taiwanese *S. latouchii*

To summarize, the order of phylogenetic isolation events for *S. latouchii* in Taiwan began with 1) clade E+P becoming isolated from western populations due to the uplift of the Central Mountain Range (2.50 Ma), followed by 2) clade W separating from Chinese populations due to the isolation of Taiwan Strait (2.32±0.32 Ma), 3) clade N separating from Chinese populations and migrating into Taiwan (2.22±0.36 Ma), 4) sub-clade P separating from sub-clade E (0.59±0.18 Ma), and 5) finally the populations of clade W starting to diverge into two possible sub-groups. The common ancestor of *S. latouchii* in Taiwan was presumably distributed widely in the lowlands of central Taiwan, both on the western and eastern sides of the young Central Mountain Range, and on the temporary land bridge across the Taiwan Strait during the Ice Age. It is interesting that initially this species did not occupy habitats in northern Taiwan, where we did not find relict haplotypes of older lineages. We suggest that the first *S. latouchii* population migrated into Taiwan through Miaoli, maybe through Shihtan (site 7), near the Holong River. The pioneers spread south- and eastward instead of northward, and formed clades E+P and W. During that period, northern Taiwan was absent from *S. latouchii* for unknown reasons. About 2.22±0.36 Ma another incursion event happened, and clade N spread widely into northern Taiwan. We have no valid molecular evidence on the first incursion route into Taiwan. In our study, no specimens were found in the nearest region of mainland China, which might have provided the key to establishing the relationship between the two incursion events. The TCS analysis failed to establish the relationships between major clades based on all haplotypes in this study, but the phylogeographic results implied the possibility of this scenario. Another nested clade analysis, a minimum spanning network (Fig. 3), indicated that the four major clades were connected in the order of clade E+P, W, C, and N. This agrees with the order of phylogenetic isolation events for *S. latouchii* listed above. The large variation between *S. latouchii* clades (32–35 bp) suggests that no gene flow has occurred recently between the major biogeographic districts of Taiwan (including, of course, China).

Biogeographic characters compare to other animals in Taiwan

Clade E+P of *S. latouchii* in Taiwan first separated from clades W, N, and C. This implies that during the formation of Taiwan Island, the Central Mountain Range first served as a genetic barrier between *S. latouchii* populations. Similar results were also found in studies of the cricket *Loxoblemmus appendicularis* (Yeh *et al*., 2004) and the primary cyprinid fish, *Varicorhinus barbatulus* (Wang *et al*., 2004). An RFLP study of *Japalura* lizards throughout Taiwan also pointed out the importance of the Central Mountain Range in forming the Taiwanese fauna, by emphasizing the presence of east–west genetic differences amongst the populations studied (Chang and Liu, 1997). The present study suggests a boundary between clades N and W close to the Miaoli Plateau. The Miaoli Plateau is another geologic barrier considered important to the phylogeography of the Taiwanese fauna (Creer *et al*., 2001; Wang *et al*., 2004; Watanabe *et al*., 2007). In addition, the

Hengchun Peninsula region has distinctive haplotypes of *S. latouchii*, as was similarly found for freshwater crabs (Shih *et al*., 2006) and amphidromous freshwater fish communities (Cheng and Fang, 1999).

Based on the phylogeographic history of *S. latouchii* within Taiwan, four biogeographic districts have been demarcated: Northern, Western, Eastern, and Hengchun Peninsula (Fig. 1). Furthermore, our data also detected a possible geographical barrier, the Chousui River, dividing the Western District into the western-middle and western-southern parts. These divisions are similar to those seen for native freshwater fishes (Tzeng, 1986; Cheng and Fang, 1999; Wang *et al*., 2004). Cheng and Fang (1999) inferred from the components of the Taiwanese freshwater fish community that there are five biogeographic districts: Northern, Middle (similar to the western-middle part of the Western District in our study), Southern (similar to the western-southern part of the Western District), Eastern, and Western Hengchun Peninsula (similar to our Hengchun Peninsula District). Although there are some disagreements on exact localities of the boundaries between districts, these two hypotheses of faunal biogeography are highly congruent. Primary freshwater fishes provide excellent opportunities for historical biogeographic studies because of their limited dispersal abilities in freshwater systems (Watanabe *et al*., 2007). However, their biogeographic information is also restricted by their original distribution, and limited interaction with the environment occurs during dispersals. In the case of freshwater fishes in Taiwan, the putative ancient river systems crossing the land bridge over the Taiwan Strait during the Pleistocene Ice Ages had the most important effect in determining components of the modern Taiwanese freshwater fauna (Tzeng, 1986; Cheng and Fang, 1999). Amphibians, such as *S. latouchii*, might have a history of invasion into Taiwan similar to that that inferred for freshwater fishes, because of their larval-aquatic life history, which has caused them to remain in close relationships with freshwater environments. Nevertheless, their adults, with motility on land, would need also to adapt to terrestrial habitats. Such multilevel interaction with the environment means that the phylogeography of amphibians may sensitively and accurately report the changes, succession, and evolution of geographic habitats.

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