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Intraspecific Relationships of Populations of the Brown Frog *Rana sauteri* (Ranidae) on Taiwan, Inferred from Mitochondrial Cytochrome *b* Sequences

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We studied the phylogenetic relationships among populations of *Rana sauteri* using partial sequences of the mitochondrial cytochrome *b* gene from 244 samples from 29 localities in Taiwan. We detected 77 haplotypes among these sequences. The phylogenetic trees contained five distinct lineages: the northern (NL), eastern (EL), southern hill (SHL), northern mountain (NML), and southern mountain (SML) lineages, defined by geographical distribution. The lineage phylogeny did not support the two-species hypothesis inferred from larval morphology. To describe the possible colonization history of *R. sauteri* in Taiwan, we propose hypotheses of within-island differentiation and a multiple-invasion model. Using a molecular clock, we estimated the order of divergence times between lineages in order to test the migration hypothesis. The multiple-invasion model was well supported by the phylogeny and a nested clade network.

Key words: *Rana sauteri*, mtDNA, cytochrome *b*, phylogeography, Taiwan

INTRODUCTION

Taiwan is a subtropical island located between 21°53'N and 25°37'N, with mountains up to 3952 m high. On this large island (3.6×10^4 km^2), the complicated geographic and climatic environments contain an amphibian fauna consisting of both Oriental and Palearctic elements (Zhao and Adler, 1993). The various periods of isolation from and connection with the Asian mainland throughout Taiwan's geographical history, and the topographic, climatic, and ecological diversity of the island, provide excellent opportunities for comparing contemporary phylogeographical patterns with biogeographical hypotheses.

Rana sauteri (family Ranidae) was initially described from Kanshirei (=Quantzelin) Village, Taiwan (Boulenger, 1909), and inhabits hilly and mountainous areas up to 3500 m throughout almost the entire island (Lue et al., 1990; Chou and Lin, 1997b). It is catalogued in the brown frog complex, which originated in the temperate zone and prefers a cold climate (Frost, 1985; Chou and Lin, 1997b). It exhibits distinct pre- and post-breeding migrations, and aggregates in numbers in streams for breeding. Reproductive groups make few calls during mating in short breeding periods. However, parts of its life history, such as information on distribution and movement routes outside the breeding season, are not well known.

The distributional range of *R. sauteri* was reported to include Guangxi Province, China (Tian and Jian, 1986; Zhao and Adler, 1993). Unfortunately, no specimens of *R. sauteri* from China were located for examination. Smith (1921) described the Vietnamese population as a subspecies, *R. s. johnsih*, that had previously been identified as a valid species, *R. johnsi* (Inger et al., 1999). Orlov et al. (2002) cataloged a possible Sauter's frog from Guangxi, China, as *R. johnsi*. Although those surveys provided information that *R. sauteri* is probably endemic on Taiwan, the taxonomic status of this species both outside and within Taiwan remains unclear.

Rana sauteri has unique adaptations to larval life in mountain streams in terms of both behavior and morphology. Tadpoles of *R. sauteri* always appear in habitats with fast running water and have a relatively depressed body, an enlarged oral disc with increased rows of labial teeth, an abdominal sucker, and robust caudal muscle and lower fins (Chou and Lin, 1997b, c). No tadpoles like this have been reported among other brown frogs in the genus *Rana* (Kuramoto et al., 1984). On the basis of the unusual larval behavior and morphology of *R. sauteri*, Fei et al. (1990) moved this species from the brown frog complex to genus *Pseudorana*. Dubois (1992), while fundamentally agreeing, considered *Pseudorana* as a subgenus of *Rana*. Furthermore, *R. sauteri* was elected as the type species of *Pseudoamolops* because of its unique larval morphology (Jiang et al., 1997). However, molecular data rejected the validity of the generic/subgeneric status of *Pseudorana* (Tanaka-Ueno et al., 1998; Matsui et al., 2001; Che et al., 2007). Tanaka-Ueno et al. (1998) inferred from cytochrome *b* sequences that *R. sauteri* and other brown frogs actually comprise a monophyletic group; other studies using 12S

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and 16S rRNA sequences (Matsui et al., 2001; Che et al., 2007) suggested this species should be placed in *Rana* as a brown frog.

Chou and Lin (1997a, b) made taxonomic revisions based on population variation in morphology in *R. sauteri*. They described frogs from the middle and eastern parts of the Central Mountain Range (CMR) as a distinct species, *R. multidenticulata* Chou and Lin, 1997, and considered populations of *R. sauteri* (sensu stricto) to be confined to the western lowlands (Chou and Lin, 1997a). These two species, however, have a wide intergradation zone along the western side of the CMR, and are difficult to discriminate on the basis of adult morphology (Chou and Lin, 1997a, b; Tanaka-Ueno et al., 1998). Tanaka-Ueno et al. (1998) studied population diversity in *R. sauteri* (sensu lato) using molecular methods. Their study detected two lineages, but these were dissimilar to the lineages inferred by Chou and Lin (1997b) on the basis of larval morphology, suggesting that Taiwanese populations, including those from the type localities of *R. sauteri* (Quantzelin) and *R. multidenticulata* (Dayuling; Chou and Lin, 1997a), should be analyzed in greater detail to understand the discordance between morphology and phylogeny. Reevaluation of the validity of *R. multidenticulata* is necessary.

Mitochondrial (mt) DNA sequence data have several benefits for studying intraspecific population structure (Avise, 1994, 2000). Recently the mitochondrial cytochrome *b* gene has successfully been used to estimate both phylogenetic relationships and population structures in various animals in Taiwan representing a wide range of divergence times (Tanaka-Ueno et al., 1998; Creer et al., 2001; Cheng et al., 2005; Yuan et al., 2006; Che et al., 2007; Jang-Liaw et al., 2008). Molecular investigations serve as a possible way to reconstruct the phylogenetic or phylogeographic patterns of species. We sought to determine intraspecific relationships using molecular data through a complete population survey at the DNA level. In focusing our attention on taxonomic problems, we chose the same sequence segment as Tanaka-Ueno et al. (1998), part of the cytochrome *b* gene. The main purpose of the present study was to clarify the genetic population structure and phylogeographic pattern of *R. sauteri* in Taiwan. A better understanding of the phylogenetic relationships of *R. sauteri* within Taiwan would be helpful in discussing the structure and formation of Taiwanese *R. sauteri* populations, clarify the taxonomic problems described above, and identify highly divergent populations of. *R. sauteri*.

MATERIALS AND METHODS

Specimen collection and DNA extraction

Adult Sauter's frogs are best sampled when they aggregate for breeding time, but collecting the tadpoles is easier than collecting adults. The *R. sauteri* specimens used in this study were collected as either tadpoles or frogs during the breeding season from 2000 to 2002. Information on the collection sites is listed in Table 1. Tadpole specimens were placed in 95% alcohol as soon as possible after they were caught. We took muscle tissues from the legs of frog specimens before placing the latter into 95% alcohol or 10% formalin. Genomic DNA was isolated from a piece of muscle tissue (about 5 mg) from either the tail (tadpoles) or leg (adult frogs) by using a standard phenol/chloroform extraction protocol (following Kocher et al., 1989) and the instructions of the Sigma GenElute Mammalian Genomic DNA Miniprep Kit (Sigma G1N70). Tadpoles (from the same clutch as the individuals analyzed) and frogs are deposited in the National Museum of Natural Science (NMNS), Taichung, Taiwan. We determined sequences from a total of 244 individuals of *R. sauteri* collected from 29 locations (GenBank EU034930–EU035174), and (with the same methods) from two other frogs used as outgroup taxa: *R. johnsi* (RJ; EU035175) and *R. longicrus* (RL; EU035176) . We obtained the sequence from GenBank for *R. nigromaculata* (RN; AB043889; Sumida et al., 2001), used as a third outgroup taxon.

DNA amplification and sequencing

GenTaq DNA polymerase (Hopegen Biotechnology Develop-

Table 1. List of sampling localities (numbers plotted in Fig. 1), sample sizes (*n*), haplotypes, lineages, elevations, and specimen numbers for *Rana sauteri* included in this study. The "Specimens" column also indicates the stage (F, frog; T, tadpole) of the animals sampled. *, Collection of the National Museum of Natural Science, Taichung, Taiwan. **, uncataloged specimens.

Locality (code)	*n*	Haplotypes (no. of individuals)	Lineage (s)	Elevation (m)	Specimens (NMNS*)
1. Wulai (WL)	10	1(6), 2(2), 3, 4	NML, NL	300	**(F)
2. Luofu (LF)	10	1(8), 2, 5	NML	300	**(F)
3. Cingcyuan (CC)	8	11, 12(2), 13, 14, 15(2), 16	NL	850	16353–16360 (F)
4. Taian (TA)	10	15(4), 31, 32, 33(2), 34(2)	NML, NL	600	15156 (T)
5. Sanyi (SY)	10	12(2), 15(4), 35, 36, 37(2)	NL	300	15124 (T)
6. Heping (HP)	10	2(6), 34, 38, 39, 40	NML	600	15001–3 (F), 15138 (T)
7. Dakeng (DK)	10	2(3), 41, 42(3), 43, 44, 45	NML	250	15123 (T)
8. Owanda (OWD)	4	2, 47, 48, 49	NML	1200	**(T)
9. Wufong (WF)	4	34(2), 44, 46	NML	200	15016–15019 (F)
10. Chungliao (CI)	4	46, 50, 51, 52	NML	200	15155 (T)
11. Hsitou (HT)	10	55(6), 56(4)	SHL	1200	**(T)
12. Tungfu (TF)	6	2(3), 48, 53, 54	NML	800	15109 (T)
13. Meishan (MS)	10	46, 55(9)	NML, SHL	200	15122 (T)
14. Lijia (LJ)	9	55(3), 56(2), 57(2), 58, 59	SHL, SML	1100	15111–15119 (F)
15. Tsuko (TK)	10	55(4), 56(5), 60	SHL	200	15131 (F), 15132 (T)
16. Quantzelin (QZL)	10	56(3), 58(2), 61, 62, 63(2), 64	SHL	300	15140–15149 (F)
17. Meishan 2 (MS2)	5	57(4), 69	SML	1000	15150–15154 (F)
18. Sanmin (SM)	10	56(2), 57(2), 58, 59, 61(3), 65	SHL, SML	600	15136 (T)
19. Tengchi (TC)	8	56(5), 57, 59(2)	SHL, SML	1100	15137 (T)
20. Liaoguei (LG)	10	56(4), 66(6)	SHL	300	15121 (T)
21. Wutai (WT)	3	56, 67, 68	SHL, SML	800	15126–15128 (F)
22. Wulu (WU)	10	76(9), 77	EL	800	**(T)
23. Minli (ML)	10	70(4), 71, 72, 73, 74(2), 75	EL	300	**(T)
24. Dayuling (DYL)	10	2(5), 15, 21, 30, 31(2)	NML, EL, NL	2600	15135 (T), 16343–16348 (F)
25. Loshao (LS)	10	21(6), 26, 27, 28, 29	EL	1200	15130 (T)
26. Wuling (WI)	8	2(5), 23, 24, 25	NML	1800	**(T)
27. Nan-ao (NA)	5	1, 2, 17, 18, 19	NML	300	15010–15014 (F)
28. Taipingshan (TPS)	10	1(4), 2(2), 5, 20, 21, 22	NML, NL	2000	15133 (T)
29. Mingchr (MC)	10	1(2), 2(3), 6, 7, 8, 9, 10	NML	1100	15110 (T)

ment Enterprise, Taiwan) and two oligonucleotide primers were used for PCR amplification of an approximately 620-bp fragment of the cytochrome *b* gene, basically following the methods of Saiki (1990). Primers L14850 (5'-TCTCATCCTGATGAAACTTTGGCTC-3') and H15502 (5'-GGATTAGCTGGTGTGAAATTGTCTGGG-3') designed by Tanaka-Ueno et al. (1998) were used to amplify and sequence a 585-bp segment of the cytochrome *b* gene corresponding to sites 16662–17804 in the *R. nigromaculata* complete mitochondrial genome sequence (Sumida et al., 2001). A modified primer, H15502M (5'-ATTAGCTGGTGTAAAATTGTCTG-3'), was used to sequence several of the PCR products. Thermal cycling conditions were 35 cycles of 95°C for 1 min, 50°C for 110 s, and 72°C for 2 min (modified from Saiki, 1990). Each round of PCR was preceded by a hot start at 95°C for 10 min to improve the yield. All PCRs were controlled with RoboCycler Gradient 96 (Stratagene). PCR products were purified with a PCR-M Clean Up System Kit (Viogene) and sequenced directly by using multiple fluorescent dyes and an Applied Biosystem 377 automatic sequencer. Sequences were aligned with the aid of MegAlign vers. 4.0 (DNA Star) and by eye. Preliminary phylogenetic and molecular evolutionary analyses were conducted with MEGA vers. 4.0 (Tamura et al., 2007) and DNA SP vers. 4.50 (Rozas et al., 2003).

Data analysis

We constructed phylogenetic trees using neighbor-joining (NJ), maximum-parsimony (MP), and maximum-likelihood (ML) analyses, all performed with PAUP*4 beta (Swofford 2001). Likelihood settings from the best-fit model (TrN+I+G) were selected by the Akaike information criterion (AIC), with the gamma distribution shape parameter=1.4829 and the proportion of invariable sites=0.5946, obtained with MODELTEST 3.7 (Posada and Crandall, 1998). Only unique haplotypes were included in the analyses; duplicate haplotypes were excluded to reduce computational time. An NJ tree was constructed using the probability model identified above, with ties broken randomly. The MP and ML analyses were conducted using random-addition heuristic searches with tree-bisection-reconnection (TBR) and subtree pruning-regrafting (SPR) branch swapping, respectively. Bootstrapping [1000 replicates for NJ (NJ option) and MP (fast heuristic search), and 100 replicates for ML (full heuristic search, TBR branch swapping)] was performed to obtain a relative measure of nodal support for the resulting tree (Felsenstein, 1985). All sites were weighted equally.

To investigate genetic interactions among *R. sauteri* populations, we constructed a minimum spanning network for all haplotypes, using MINSPNET (Excoffier and Smouse, 1994) to link the haplotypes in a hierarchical manner based on variation between sequences. After linking the haplotypes, we grouped them into higher clades. Clades with closer relationships were further linked to each other to form a network. The number of mutations between haplotypes in pairwise comparisons was calculated by using MEGA vers. 4.0 (Tamura et al., 2007). The selective neutrality of all sequences was assessed by Tajima's *D* (Tajima, 1989) statistic and the *D* and *F* statistics of Fu and Li (1993) within each lineage and population locality.

To estimate divergence times between lineages, we used LINTREE (Takezaki et al., 1995) to conduct a two-cluster test of constancy of evolutionary rates based on K2P distances among all haplotypes. The test employed the NJ method to establish the tree topology. We also used *R. johnsi*, *R. longricus*, and *R. nigromaculata* as outgroups in this test. The height of the branch point of two lineages was calculated and defined as half the average of the mean nucleotide distance between the two lineages. The divergence time between lineages was estimated by the ratio of the height to the divergence rate. We applied the divergence rate of 1.41%/million years (Myr) inferred by Jang-Liaw et al. (2008) in a molecular study on the population diversity of the Taiwanese frog *Sylvirana latouchii*.

RESULTS

Lineages and their geographic distributions

Partial cytochrome *b* sequences 585 bp long amplified from 244 *R. sauteri* individuals yielded 77 unique haplotypes. Relationships between sequences and haplotypes are listed in Table 1. We treated haplotypes, instead of individual sequences, as operational taxonomic units (OTUs) to examine the phylogenetic relationships among *R. sauteri* populations, with the aligned sequences containing no gaps or missing data, i.e., no insertions, deletions, or stop codons were found within *R. sauteri* or in the outgroup species.

Tree topologies consistently rejected the monophyly of *R. sauteri* populations, with bootstrap values of 100 for NJ, 100 for MP, and 100 for ML . The analyses supported the presence of two major lineages within *R. sauteri*. Furthermore, haplotypes of *R. sauteri* grouped into five detailed lineages. One of the major lineages was defined as the "lineage-1 complex", which included 4 minor lineages: 1-1 to 1-4. The distribution of the lineage-1 complex covered almost all collection localities, except for Chinchun (site 3). The second major lineage, designated as lineage 2, was distributed in northern and north-central Taiwan. The southern boundary of lineage 2 was from Sanyi (site 5) to Dayuling (site 24).

The geographical distributions of these lineages are shown in Fig. 1. The distributions of *R. sauteri* lineages were

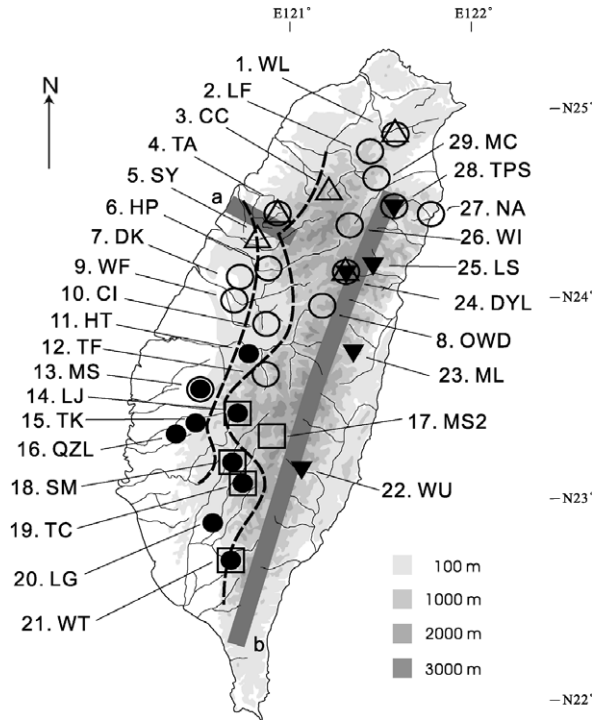

Fig. 1. Map of Taiwan showing sampling locations and haplotype patterns for *Rana sauteri*. See Table 1 for locality information. Open circles, northern mountain lineage (NML); open squares, southern mountain lineage (SML); solid circles, southern hill lineage (SHL); solid reversed triangles, eastern lineage (EL); open triangles, northern lineage (NL) (refer to Fig. 2). The dashed lines indicate putative borders between the zoogeographical ranges of larval *R. sauteri* proposed by Chou and Lin (1997a, b). Bold gray line "a" indicates the location of the Miaoli Plateau; "b" refers to the Central Mountain Range.

correlated with topographic features. We named these lineages by their geographical distribution, e.g., lineage 2 as the "northern lineage" (NL) because the populations were limited to northern and north-central Taiwan. In the lineage-1 complex, lineage 1-1 was named the "northern mountain lineage" (NML), distributed in mountain areas north of Meishan (site 13). It was geographically distinct from lineage 1-2, the "southern mountain lineage" (SML), which was spread along the southwestern mountains from Lijia (site 14) to Wutai (site 21). Although the NML and SML were not supported by high bootstrap values, their divergence in the NJ and MP trees was clear. The NML and SML share most of the mountainous areas of Taiwan, and together comprise the "pan-mountain lineage" (PML). The boundary between these two sub-lineages is between Meishan (site 13; inhabited by the NML) and Lijia (site 14; inhabited by the SML). No site was found which contained both NML and SML individuals.

Lineage 1–3 was spread throughout southwestern Taiwan. The elevations of the collection localities for this lineage were lower than those of the SML on average, so it was designated the "southern hill lineage (SHL)". The SHL overlapped with the PML in some sampling localities, such as sites 13 and 14, containing the NML and SML, respectively, but not with lineage 1–4. Lineage 1–4 was only distributed on the eastern side of Taiwan and was designate the "eastern lineage" (EL). All samples in this lineage were collected from five collecting sites; four of which were located on eastern side of the CMR, with only site 24 (Dayuling) within the CMR at a high elevation, 2600 m.

Phylogenetic analysis

Nucleotide sequence distances among *R. sauteri* haplotypes ranged from 0.17–5.40% (data not shown). The largest distance was detected between haplotypes 37 (which appeared in Sanyi, site 5) and 38 (which appeared in Heping, site 6), with base substitutions at 30 sites. Distances between lineages were rather small, 1.11–4.46% (Table 2). The overall mean distance among all haplotypes was 0.0230±0.0040 (mean±SE).

Among the 77 haplotypes of Taiwanese *R. sauteri* there were 85 polymorphic sites, 49 of which were parsimony-informative. The MP analysis revealed 678 equally parsimonious trees (340 steps) with a consistency index (CI) of 0.5500, a retention index (RI) of 0.8723, and a rescaled consistency index (RC) of 0.4798. The NJ, MP, and ML analyses provided largely congruent tree topologies, with proportionally similar bootstrap values supporting major lineage divergences (Fig. 2). The outgroup taxa, *R. longicrus*, *R. johnsi*, and *R. nigromaculata*, were separated from the *R. sauteri* clade in that order. The mean distances between them and *R. sauteri* populations were 0.1858±0.0190, 0.1995±0.0203, and 0.2127±0.0214, respectively.

All collection sites had more than one haplotype (Table 1), but most sites (20 of 29) contained only one lineage. Eight sites contained two lineages. Site 24 (Dayuling) contained three lineages: the NL, NML, and EL. Haplotype diversities (Hd) within the NML and EL were slightly higher than those of the other lineages (Table 3). The EL showed the highest nucleotide diversity (π) value, which was 1.375 times as high as that within the NML, and about twice as high as for the other lineages. The NML had the largest sample size, and the most haplotypes and polymorphic

Table 2. F*st* values inferred from all cytochrome *b* sequences (above diagonal), and estimated pairwise K2P distances (below diagonal) based on unique cytochrome *b* haplotypes, between lineages of *Rana sauteri* (see Fig. 2 for abbreviations). The column to the right of the matrix shows within-lineage distance values inferred from the haplotypes.

	NML	SML	SHL	EL	NL	d average within lineages
NML		0.72427	0.77375	0.86489	0.92308	0.00593
SML	0.0121		0.77615	0.87851	0.87851	0.00412
SHL	0.0141	0.0111		0.86587	0.93562	0.00458
EL	0.0339	0.0311	0.0285		0.90009	0.00609
NL	0.0446	0.0409	0.0416	0.0412		0.00459

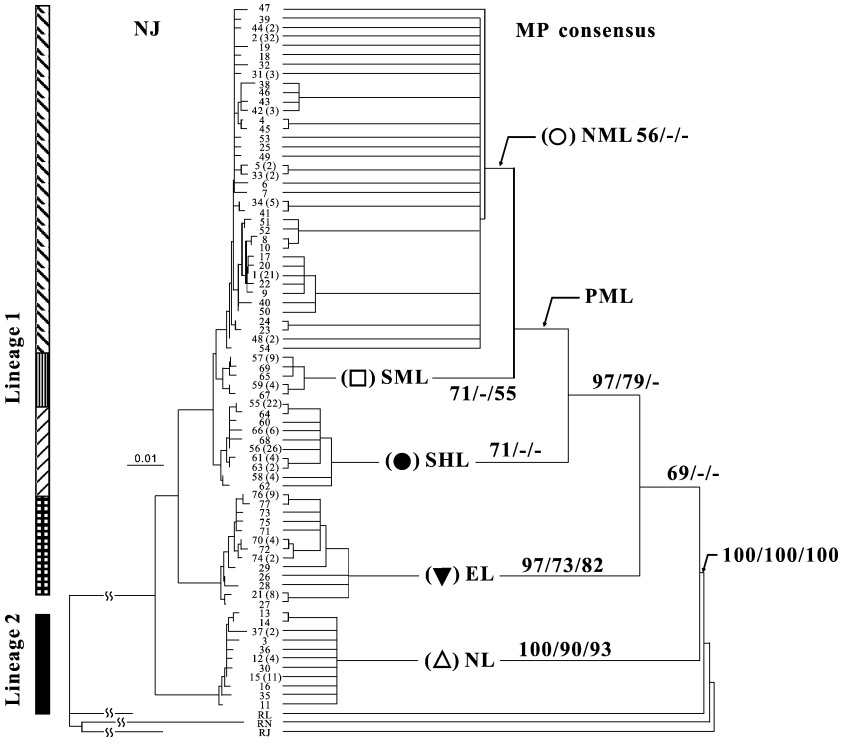

Fig. 2. Neighbor-joining (NJ, left) and maximum-parsimony consensus (MP, right) trees for *Rana sauteri* cytochrome *b* haplotypes. Near branches are bootstrap values (NJ/MP/maximum likelihood) for the adjacent nodes. Lineages are represented by symbols corresponding to those in Fig. 1: open circles, NML; open squares, SML, solid circles, SHL, solid reversed triangles, EL; open triangles, NL. Haplotypes are shown as OTUs identified by number, followed in parentheses by the number of individuals in which a haplotype occurred, if more than one. The lineage abbreviations are defined in the caption to Fig. 1. The scale bar to the left of the NJ tree indicates branch lengths for the NJ tree, in substitutions per site.

sites. There were 2.57–7.2 times as many polymorphic sites in the NML as in the other lineages.

Neutrality tests

Values of the neutrality test statistics are shown in Table 4. Test values did not significantly deviate from zero in most localities, except at Heping (site 6) and Meishan (site 13). In these two localities, all three test values (Tajima' *D*; Fu and Li's *D* and *F*) significantly deviated from zero. However, the neutrality test values among lineages showed deviations that were more significant. All three test values for the NML and Tajima' *D* for the NL deviated significantly from zero. Furthermore, two tests (Fu and Li's *D* and *F*) including all specimens showed significant deviations from zero.

Minimum spanning network and phylogeographic information

The minimum spanning network was helpful in understanding the structure of phylogenetic relationships within haplotypes of *R. sauteri* among populations (Fig. 3). In this network, five lineages were clearly separated, which agreed with the NJ tree. The NL, which comprised 11 haplotypes among 25 samples from five collecting sites (Table 3), contained 19 mutational differences from the lineage-1 complex. Haplotype 15 was the major haplotype (represented by 11 individuals) in the NL and was distributed in all five localities. Other haplotypes of the NL were distributed in one or two localities and among no more than four individuals. The NL also showed the highest *F*st values (0.87851–0.93562) compared to the other lineages. The haplotype diversity (Hd) of the NL was high (0.793; see Table 3), but the nucleotide diversity (π) was relatively low (0.275) compared to the other lineages.

In the lineage-1 complex, variation was low among the NML, SML, and SHL, with no more than four mutational differences. The EL contained 13 mutational differences from the lineage-1 complex. There were 13 haplotypes in the EL, among 32 individuals from five collecting sites. Haplotypes 26 and 71 constituted distinct phylogeographic centers in the EL, but each was occurred in only a single individual. The most widespread haplotype in the EL was haplotype 21, which occurred in eight individuals from localities (sites 24, 25, and 28). Haplotype 76 was found in the most individuals (nine) in this lineage, all from site 29 (Wulu). Other haplotypes in the EL were represented by no more than two individuals and appeared at only one collecting site. *F*st values between the EL and other lineages of the lineage-1 complex were high, 0.86489–0.87851. The Hd value for the EL was also high (0.857), lower only than that for the NML (0.861), while the π value for the EL was the highest (0.506) among all lineages.

The SHL occupied the internode position between the EL and PML. This lineage comprised 10 haplotypes represented by 68 individuals from nine localities. Haplotypes 56 and 55 were represented by 26 and 22 individuals, respectively, and the former occurred at all SHL collecting sites except site 13 (Meishan). Another eight haplotypes in the SHL were represented by 20 individuals. The Hd and π values for the SHL were both the lowest (0.744 and 0.220, respectively) among *R. sauteri* lineages. *F*st values among the SHL and PML were lower than those mentioned above; they were 0.77375 and 0.77615 for the NML and SML, respectively.

Within the PML, there were four nucleotide differences between the districts of the NML and SML. The NML was the largest lineage, with 38 haplotypes among 103 individuals from 15 collecting sites. This high sample size gave this lineage the highest Hd value (0.861) among lineages, but the π value (0.368) was lower than that for the EL. In the NML, haplotype 2 was the major haplotype (represented by 32 individuals) and occurred at 11 localities. Haplotype 1 was the second most abundant haplotype (represented by 21 individuals) in the SML in terms of sample size, but it was limited to five sampling localities in northeastern Taiwan. These two haplotypes occurred in roughly half (53/103) the individuals sampled in the NML.

The SML was smaller than the NML. Only five haplotypes occurred

Table 3. Intra-lineage variation for phylogroups based on cytochrome *b* sequences in *Rana sauteri* on Taiwan. Therre are five lineages, as outlined in Fig. 2. Standard deviations are in parentheses.

Lineage	Sample size	No. of haplotypes	No. of polymorphic sites	Haplotype diversity (Hd)	Nucleotide diversity (π) in percent
NML	103	38	36	0.861 (0.026)	0.368 (0.026)
SML	16	5	5	0.650 (0.108)	0.221 (0.044)
SHL	68	10	11	0.744 (0.035)	0.220 (0.025)
EL	32	13	14	0.857 (0.041)	0.506 (0.032)
NL	25	11	13	0.793 (0.076)	0.275 (0.054)
Total	**244**	**77**	**85**	**0.950 (0.006)**	**1.847 (0.098)**

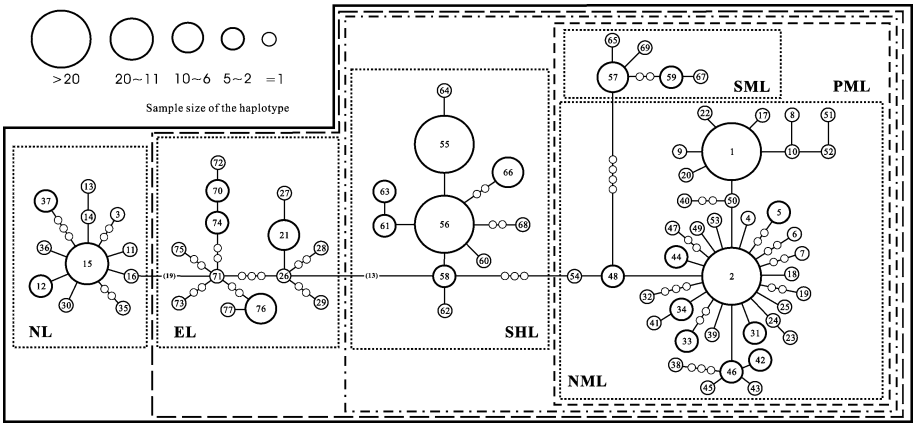

Fig. 3. Minimum spanning network for cytochrome *b* haplotype sequences from populations of *Rana sauteri*, generated with MINSPNET (Excoffier and Smouse, 1994). The numbers inside circles designate haplotypes. Each line connecting two circles represents a single mutational step between one haplotype and another. The size of the circles is proportional to the number of individuals observed having a particular haplotype. The smallest circles, without numbers, represent intermediate haplotypes that were not actually observed.

among 16 individuals from five sampling localities. Haplotype 57 was the major haplotype, represented by nine individuals from four localities of the SML, except in the southernmost sampling site (site 21, Wutai). Haplotype 59 occurred in four individuals from three localities. Other haplotypes (65, 67, and 69) were represented only by single individuals. The *F*st value between these two lineages was the smallest (0.72427) in the study; no individuals from the NML and SML overlapped in distribution.

Some *R. sauteri* haplotypes were important in the number of individuals in which they occurred and by being key structures of the nested clade network, such as being located at interior nodes. Haplotypes 15, 76, 56, 2, and 57 were the major haplotypes in terms of abundance in the NL, EL, SHL, NML, and SML, respectively. In addition, some other haplotypes were important due to their positions between lineages, e.g., haplotypes 16, 26, 58, 54, and 48.

Table 4. Neutrality test statistics for *Rana sauteri* by collecting locality and lineage, based on the total mutations in partial cytochrome *b* sequences. *n*, numbers of sequences; *, statistically significant at the 5% level; **, statistically significant at the 2% level; n/c, not calculated because sample size too small (fewer than four sequences); dashes (–), not calculated due to lack of polymorphism in the data.

Locality-Lineage	*n*	Tajima's *D*	Fu and Li's *D*	Fu and Li's *F*
1. WL	10	–1.66328	–2.00432	–2.16538
2. LF	10	–0.82229	–0.33833	–0.5109
3. CC	8	–0.7554	–0.49407	–0.61102
4. TA	10	1.84164	1.13785	1.48166
5. SY	10	–0.77402	–0.12602	–0.3205
6. HP	10	–1.90129*	–2.21874**	–2.40603**
7. DK	10	–0.77402	–1.13149	–1.17165
8. OWD	4	–0.78012	–0.78012	–0.72052
9. WF	4	0.16766	0.16766	0.14992
10. CI	4	–0.06501	–0.06501	–0.06004
11. HT	10	1.30268	0.80424	1.02604
12. TF	6	–0.44736	–0.37481	–0.41015
13. MS	10	–1.87333*	–2.18183*	–2.36585*
14. LJ	9	1.02379	0.92083	1.05421
15. TK	10	0.1203	–0.2802	–0.20464
16. QZL	10	–0.45258	–0.36755	–0.43576
17. MS2	5	–0.8165	–0.8165	–0.77152
18. SM	10	1.00846	0.51295	0.71351
19. TC	8	1.02206	0.87595	1.00406
20. LG	10	1.64145	1.02623	1.30973
21. WT	3	n/c	n/c	n/c
22. WU	10	–1.11173	–1.24341	–1.34668
23. ML	10	–0.58262	–0.93878	–0.9551
24. DYL	10	–0.13708	0.48991	0.37636
25. LS	10	–1.27616	–1.51001	–1.62963
26. WI	8	–0.81246	–0.6323	–0.73855
27. NA	5	–0.19092	–0.19092	–0.19784
28. TPS	10	–1.42618	–1.65755	–1.804
29. MC	10	–0.5511	–0.83444	–0.85903
NML	103	–2.12535*	–4.3797**	–4.17629**
SML	16	–0.47057	–1.05227	–1.02652
SHL	68	–1.21493	–1.64264	–1.77162
EL	32	–0.49139	–1.87375	–1.6867
NL	25	–1.83289*	–2.24882	–2.47828
All specimens	244	–0.68979	–3.03902*	–2.32088*

Table 5. Results of the 2-cluster test and estimated divergence times for *Rana sauteri*, inferred from haplotype lineages (Fig. 2). For the method of divergence-time estimation, see Materials and Methods. Z, delta/SE; delta, | b1–b2 |; height, average (b1, b2); divergence time, height/divergence rate (=1.41% Myr in this study); CP value=1–*p* value.

Sister groups (1 vs. 2)	CP (%)	Z	b_1	b_2	delta	height (SE)	divergence time (mean±SE; Ma)
NML vs. SML	62.66%	0.8973	0.0076	0.0046	0.0030	0.0061 (0.0018)	0.43±0.13
PML vs. SHL	79.60%	1.2722	0.0088	0.0050	0.0038	0.0069 (0.0019)	0.49±0.13
(PML+SHL) vs. EL	39.70%	0.5234	0.0178	0.0148	0.0029	0.0163 (0.0034)	1.15±0.24
NL vs. Lineage 1	2.40%	0.0329	0.0215	0.0217	0.0002	0.0216 (0.0039)	1.53±0.28

Divergence times among major lineages within *Rana sauteri*

We conducted a 2-cluster test that included all 77 *R. sauteri* haplotypes and the cytochrome *b* sequences from the outgroup taxa, *R. johnsi*, *R. longicrus*, and *R. nigromaculata*. The results of the test are summarized in Table 5. The confidence probability (1–*p* value; CP value) was computed by the 2-cluster test. We applied a divergence rate of 1.41%/Myr following Jang-Liaw et al. (2008). The divergence time between the lineage-1 complex and lineage 2 was estimated to be 1.53 million years ago (Ma). Divergence times within the lineage-1 complex were estimated to be 0.43–1.15 Ma. The EL was the first lineage to diverge within the lineage-1 complex. These results suggest that divergence events in all lineages occurred in the Pleistocene.

DISCUSSION

Morphological variation and ecological adaptations

Taxonomic surveys suggested a possible species hidden within *R. sauteri* in Taiwan. A thorough morphological study mainly of oral disc structures of tadpoles of *R. sauteri* suggested that a valid species, *R. multidenticulata*, was distributed at higher elevations in the CMR and represented an evolutionary lineage separated from the lowland form by a narrow intergradation zone (Fig. 1) (Chou and Lin, 1997a, b). Kuramoto et al. (1984) also found different hybrid survival rates from Alishan (a mountainous area) and Kwantzuling (=Quantzelin, a lowland area) in a hybridization experiment with *R. sauteri*. This implied that there were indeed some genetic divergences between these populations, thus suggesting the possibility that the Alishan population was a cryptic species distinct from *R. sauteri*. Subsequent research on *R. sauteri* using molecular data seemed to support this conclusion: notable genetic variation occurred among populations from different elevations.

However, the considerable climatic variation and multifaceted geomorphology of Taiwan may have led to a higher possibility of local adaptations and geographical morphological variation in this species (Chou and Lin, 1997a, b). Lai et al. (2003) examined reproductive adaptations in *R. sauteri* at different elevations. At high elevations, the minimum temperature is a limiting factor for tadpole growth and survival. If the breeding season were in fall rather than spring, tadpoles of *R. sauteri* would encounter a harmfully cold winter and die off before reaching metamorphosis. On the other hand, the maximum temperature is a limiting factor for survival of those populations at low elevations. If tadpoles at low elevations were born in spring rather than fall, they would die off

due to high summer temperatures. Lai et al. (2003) concluded that adaptive shifts in *R. sauteri* tadpoles have resulted in the formation of elevational clines in the breeding season as well as in reproductive and life history traits. These authors hypothesized that variation in the breeding season among populations at different elevations was the primary adaptive mechanism in long-tern selection in *R. sauteri*. This mechanism ensures the best survival and growth of *R. sauteri* for completing the aquatic larval phase. The adaptive shifts in elevation largely agreed with the distribution patterns of *R. sauteri* and *R. multidenticulata* (Chou and Lin, 1997a). Although no study has yet been published on the connection between ecological and morphological adaptation in *R. sauteri*, it is reasonable to assume that populations at different elevations show ecologically adaptive variation that is reflected in morphology, such as divergence in the oral disc structures of tadpoles related to strategies of metamorphosis.

Phylogenetic relationships and distributions of lineages

Two major lineages including five sub-lineages (NML, SML, SHL, EL, and NL) were well differentiated and well supported in the NJ and MP trees, and in clades in the nested clade analysis (Figs. 2, 3), and were closely correlated with geographical distributions. The trees were identical in gross topology and were congruent with the MINSPNET network analyses.

The phylogenetic relationships inferred from genetic data in this study are not in accordance with the conclusions on intraspecific differentiation inferred from morphological variation in tadpoles (Chou and Lin, 1997b). The *R. sauteri* sequences grouped into five lineages that were correlated with geographical distributions, but not solely with distributions by elevation. For example, six haplotypes (56, 58, 61, 62, 63, and 64) from 10 individuals at Quantzelin (site 16; type locality of *R. sauteri*) belonged to the SHL. SHL haplotypes occurred also at higher elevations at two southwestern sites in the CMR, Lijia (site 14) and Tengchi (site 19), but were absent from the lowlands of northwestern Taiwan. On the other hand, Dayuling (site 24; type locality of *R. multidenticulata*) contained five haplotypes (2, 15, 21, 30, and 31) representing three lineages, including the NML (h2 and h31), which could be found at lower elevations such as at Luofu (site 2; 300 m in elevation), Dakeng (site 7; 250 m), and Nan-ao (site 27; 300 m). Furthermore, Dayuling (site 24) was probably historically an important geographical passage point in the migration and dispersal of *R. sauteri*. Haplotypes (lineages) found at this site were not limited to high elevations. The three lineages occurring at Dayuling also occurred widely at both high and low elevations, excluding southwestern Taiwan.

A phylogenetic study (Tanaka-Ueno et al., 1998) on the brown frog complex in Taiwan and Japan, which examined genetic relationships among *R. sauteri* populations in Wulai, Sanyi, and Alishan (geographically close to our site 14, Lijia), found that the Wulai population was genetically unique, whereas the other populations were genetically closer. In that study, only three *R. sauteri* populations (five individuals in total) were sampled, and only two *R. sauteri* lineages were identified. This result was incongruent with both the hypothesis of Chou and Lin (1997b) and our results. We reexamined these five sequences by adding them to our data matrix and then analyzed the latter with Mega vers. 4.0 (data not shown). The sequences from Sanyi and Alishan in Tanaka-Ueno et al. (1998) grouped in the NL and SHL, respectively; and the distances between the previous sequences in these two lineages and our *R. sauteri* dataset were 0.0374±0.0065 and 0.0249±0.0047 (mean±SE), respectively. However, the sequences from Wulai samples in Tanaka-Ueno et al. (1998) were quite distant from sequences in our *R. sauteri* dataset; the mean distance between them was 0.1826±0.0182, which was close to values between the outgroup taxa and *R. sauteri* populations in our study (range 0.1858–0.2127). We found no similar haplotypes in our samples.

Scenario for the dispersal of *R. sauteri* on Taiwan

We can interpret our data more clearly from the results of the minimum spanning analysis and estimates of lineage divergence times. In the network, the relative positions of haplotypes are useful in reconstructing possible dispersal routes of *R. sauteri*. Lineage divergence times can help arrange the order of possible divergence events. Furthermore, we applied the multiple-invasion model, which previous phylogenetic research on the Taiwanese fauna has supported (Yu, 1995; Yu et al., 1996; Ota, 1997; Hsu et al., 2000; Creer et al., 2001, 2004; Jang-Liaw et al., 2008), to construct a possible dispersal history for *R. sauteri*. We assume that the first *R. sauteri* population, which was the ancestor of the lineage-1 complex, immigrated into and spread over most of Taiwan about 1.53 Ma, before the CMR rose high enough to form a geographical barrier. The population then began to differentiate from 1.15–0.49 Ma due to increasing topographical impediments. During this period, the EL, SHL, and PML formed as the result of both topographical and ecological isolation. When the second invasion occurred, the dispersal of the new settlers was probably impeded by the Miaoli Plateau and was limited solely to northern and north-central Taiwan; these immigrants were the ancestors of the NL group.

In phylogeographic research, Avise et al. (1987) considered the most common haplotypes to be plesiomorphic (ancestral), and other genotypes to be apomorphic (derived). Following this assumption, haplotype 2 is plesiomorphic in the lineage-1 complex. It was distributed in northern and north-central Taiwan, including Wulai (site 1), Luofu (site 2), Mingchr (site 29), Nan-ao (site 27), Taipingshan (site 28), Wuling (site 26), Dayuling (site 24), Heping (site 6), Dakeng (site 7), Owanda (site 8), and Tungfu (site 12). These sites might have been the first areas the ancestor of *R. sauteri* occupied in Taiwan. In the SHL, haplotype 56 was widespread, occurring at 8 sampling sites (11, 14, 15, 16, 18, 19, 20, and 21). In eastern Taiwan, haplotype 21 occurred at Taipingshan (site 28), Loshao (site 25), and Dayuling (site 24); other haplotypes occurred only at single sites.

The NL is unique in being a relict of the second invasion. The NL was distributed only north of Dayuling (site 24), and its range and population size were both smaller than those of the NML. The most widely distributed haplotype in the NL was haplotype 15, found at sites 3, 4, 5, and 24. These sites are located mainly on or near the Miaoli Plateau, except for site 24 (Dayuling).

The scenario can be discussed in greater detail. *Rana sauteri* inhabited northern Taiwan after the ancestor migrated from the continental mainland across a land bridge spanning the ancient Taiwan Strait about 1.53 Ma. This population occupied north-central Taiwan and expanded eastward and southward. The eastern population was isolated by the rising CMR about 1.15 Ma, and has since differentiated genetically from the western population. Subsequently, a small population that expanded southeasterly into habitats at lower elevations experienced long-term selection by environmental factors such as temperature (Lai et al., 2003) and, about 0.49 Ma, formed the SHL. The remaining population in the western portion of the CMR was ancestral to the PML, including the ancestors of the NML and SML. These two sibling lineages began to differentiate due to geographical distance more recently, about 0.43 Ma. Within the SML, haplotype 57 was the most widespread, occurring at sites 14, 17, 18, and 19, which possibly comprised the original range of the SML. Later, a second invasion occurred. A population derived from an ancestor from the continental mainland crossed the Taiwan Strait by land bridge during a regression (e.g., glacial) period. This population occupied primarily the Miaoli Plateau and formed the NL. Nevertheless, vicariance seems to be the dominant mode of divergence in *R. sauteri*.

Unfortunately, we obtained no samples of *R. sauteri* or of sibling species phylogenetically close to *R. sauteri* from areas near Taiwan for comparative analyses. The ancestors of *R. sauteri* on the continental mainland are an important key to revealing the history of dispersal to and within Taiwan. From our migration scenario, we propose two hypotheses on the unknown continental ancestor: 1) it was once widely distributed on the southeastern Asian continent near Taiwan, but might have gone extinct in most areas after the second invasion event occurred; 2) the mutation rate of *R. sauteri* is higher on Taiwan than on the continental mainland. The population on Taiwan differentiated between the two invasion events, while at the same time, the population on the continent showed less differentiation, as reflected by the genetic structure of the NL.

Although the null hypothesis was not significantly rejected for most populations, the neutrality test results for the entire population implied that the cytochrome *b* segment might not be evolving neutrally or steadily in *R. sauteri*. Compared to the genetic structure of another ranid frog on Taiwan, *Sylvirana latouchii* (Jang-Liaw et al., 2008), *R. sauteri* has had a more complicated history and exhibits unique reproductive traits (Lai et al., 2003; Huang et al., 2004). This might be why *R. sauteri* has retained a more-complex phylogeographic structure than *S. latouchii*. Moreover, ecological traits also affected the differentiation history of *R. sauteri*. In *S. latouchii*, major divergence events occurred 2.50–2.21 Ma, much earlier than those we detected for *R. sauteri* (beginning 1.53 Ma). A possible explanation is that *R. sauteri* is adapted to higher elevations and a cooler climate. The elevation of geographical barriers had to be higher for *R. sauteri* then for other anurans on Taiwan, before they would impede the movements of the former.

We found that the Miaoli Plateau played an important role in the dispersal scenario. This area is the geographic center of distribution of the NML and was probably the original center of the NL. Wang et al. (2004) and Watanabe et al. (2007) also respectively hypothesized that this area was a center of dispersal for the endemic freshwater fishes *Varicorhinus barbatulus* (Pellegrin, 1908) and *Pseudobagrus brevianalis* Regan, 1908, respectively, on Taiwan. This implies that the ancestors of these freshwater fish migrated to Taiwan across the Miaoli Plateau. The dispersal of primary freshwater fishes follows freshwater environments such as rivers, lakes, and wetlands. The topology of the landscape, especially freshwater systems, thus should affect their migration routes. River systems should also affect the dispersal of amphibians, with aquatic larvae. From this viewpoint, we presume that freshwater fishes and amphibians will share some similar characteristics in dispersal patterns, and that the Miaoli Plateau might have been a beachhead for new immigrants ancestral to *R. sauteri*.

Chen (1993) compared genetic patterns between two Sauter's frog populations from northern and middle Taiwan related to two river systems and suggested that there was a close connection between genetic divergence and habitats in the river systems. We expected that the historical dispersal pattern of *R. sauteri*, as an amphibian breeding in specific streams, was affected by the terrestrial topography. In our opinion, *R. sauteri* is not a riparian frog that inhabits only streams; its home range broadly includes terrestrial habitats. Large geographical factors, including the terrestrial topography, microclimates, and habitat succession, have played important roles in the population evolution of *R. sauteri*.

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