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Within-island speciation with an exceptional case of distinct separation between two sibling lizard species divided by a narrow stream ☆

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ABSTRACT

Delimitation of genetic and geographic boundaries between species is a focus of evolutionary biology. In this study, we demonstrated fine-scale differentiation of *Takydromus formosanus* species complex comprising four insular endemics on Taiwan Island. Phylogeny and ancestral range reconstruction based on mitochondrial DNA sequences of 430 *Takydromus* lizards (405 lizards of this complex throughout their distribution range, and 25 lizards from 11 other species) indicated that the major branching process occurred within Taiwan, which represented a solid evidence of within-island speciation on this small island. We further demonstrated an exceptional case of a pair of sister species, *T. viridipunctatus* and *T. luyeanus*, that were separated by a narrow stream with a width of only 15 m. This pattern might be one of the narrowest contact zones ever documented in terrestrial vertebrates. To evaluate the level of genetic introgression between these sister species, a fine-scale collection of another 382 lizards was conducted along a transect line across the stream. A total of 13 microsatellite markers and mtDNA genotyping was used to detect a low proportion of hybrids (5.7–9.9% from STRUCTURE, and 2.3% from DAPC). Our results indicated that the two clades are highly differentiated across this extremely short distance.

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1. Introduction

Islands have been recognised as natural laboratories for studying the processes of species diversification (Johnson, 2003; Losos and Ricklefs, 2009). Various factors, including dispersal, vicariance and ecological adaptations, are involved in the formation of island biota (Paulay and Meyer, 2002; Cowie and Holland, 2006; Ryan et al., 2007). Owing to the obvious isolation by oceans, traditional research related to insular endemism has focused on the evolutionary process of inter-island speciation. However, within-island speciation has gained increasing attention in recent decades (Kisel and Barraclough, 2010; Rosindell and Phillimore, 2011). Several well-studied cases have been presented, especially in tropical islands such as the West Indies and Madagascar, where within-island speciation has been shown to play an important role in increasing the biodiversity on these islands (Losos et al., 1998; Rabemananjara et al., 2007; Townsend et al., 2009). However, research in other regions of the world is comparatively limited. Islands located near the eastern and northeastern Asian coastline are among the regions with relatively few studies concerning this issue.

Taiwan, a medium-sized island (36,000 km^2) located offshore from mainland Asia, is suitable for the study of evolutionary events between continents and islands because of its geographic location connecting mainland Asia and the Ryukyu Archipelago. Due to its steep topography among adjacent regions, this island served as a refugium for both high-latitude and high-altitude species during the repetitive fluctuation of global climate in past glaciations (Wu et al., 2006; Yuan et al., 2006). A number of published studies collectively indicated that the biodiversity on this island was created by three major factors: (1) a complicated and repetitive colonisation history from mainland Asia (Lin et al., 2002; Ota et al., 2002; Jang-Liaw et al., 2008; Jang-Liaw and Chou, 2011), (2) inter-island dispersals from adjacent islands (Ota, 1997, 2000), and (3) genetic differentiation shaped by high landscape heterogeneity across the island (Lin et al., 2012). Although the first two factors have been thoroughly addressed from biogeographic or phylogenetic perspectives, the last model has not yet received much attention.

In this study, we aim to demonstrate a case of speciation occurring on the island and an extremely narrow contact zone across a stream. The *Takydromus* lizards (Squamata: Lacertidae) are widely distributed in the Oriental and eastern Palaearctic regions, with the greatest diversity in Taiwan (Arnold, 1997; Lue and Lin, 2008). In most cases, lizards of this genus rarely occur sympatrically, possibly due to their overlapping ecological requirements and niche

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competition. Taiwan Island represents a unique case of coexistence of multiple *Takydromus* species, which rarely occurs in any other place in the world (Arnold, 1997; Lin et al., 2002; Lue and Lin, 2008). In 2008, *T. formosanus*, which is widely distributed throughout the island, was identified as a complex of three sibling species owing to their differences in morphology and courtship colourations. This discovery increased the species number on the island to seven, including six endemics. According to current taxonomy, *T. formosanus* (Boulenger, 1894), with no sexual dichromatism, occurs only in southwestern Taiwan. *Takydromus viridipunctatus* and *T. luyeanus* Lue and Lin, 2008 are distributed in northern and eastern Taiwan, with nuptial colouration of green and yellow spots, respectively. The other two species in this clade are *T. hsuehshanensis* Lin and Cheng, 1981, which is unique as it is the only alpine species in this genus occurring exclusively in montane grasslands, and *T. wolteri* (Fischer, 1885), which is distributed in mid-northern China, Korean peninsula, and southernmost Russian Far East thus forming a disjunctive distribution from other closely related species. With their well-defined and parapatric distribution pattern, this group of lizards provides an ideal target to investigate the tempo and mode of the speciation process in East Asian islands.

Discussing distributional and genetic boundaries among these closely related species is the second goal of this study. The boundary between sister species, usually known as contact zones or hybrid zones (Barton and Hewitt, 1985; Harrison, 1993), have long been recognised as "windows on the evolutionary process" (Harrison, 1990), which provide valuable chances to detect selective adaptation and gene flow between newly evolving taxa (Abbott et al., 2013; Hoskin and Higgie, 2013). The formation and maintenance of a contact zone relies on a number of mechanisms that are related to ecological differentiation or reproductive isolation. If hybridisation does not increase fitness of offsprings, a species will tend to avoid mating with other species to reduce energy costs. Reproductive character displacement or reinforcement, referred to as the strengthened performance of courtship signals, will have a chance to evolve from the contact region between sister taxa (Howard, 1993; Noor, 1999; Servedio and Noor, 2003). In contrast, hybridisation and subsequent genetic introgression contribute to homogenise their differences (Rieseberg et al., 1999; Pfennig, 2003; Turner et al., 2005). The future of such a contact zone may have several possibilities: extinction of one of the two populations, stable coexistence with hybridisation, fusion of the two populations, or an increase in premating divergence that facilitates the formation of distinct species (Barton and Hewitt, 1985). Research across the contact region has the potential to provide insights into speciation processes, including the process of differentiation and maintenance of this barrier (Abbott et al., 2013; Hoskin and Higgie, 2013).

This study focuses on three major issues among this group of lizards: within island speciation, fine-scale genetic differentiation, and the detection of hybridisation. The specific purposes of this study were as follows: (1) to provide evidence for within-island speciation using molecular phylogeny and ancestral range reconstruction; (2) to reveal fine-scale differentiation between *T. viridipunctatus* and *T. luyeanus* that are separated by a narrow stream; (3) to evaluate the ratio of hybrids and the level of genetic introgression in this sister pair; and (4) to discuss this contact zone from geographic and genetic viewpoints.

2. Materials and methods

2.1. Sample collection and DNA extraction

During 2002–2003, 402 samples belonging to the *Takydromus formosanus* species complex were collected from 34 localities throughout Taiwan (Fig. 1B; Table S1). This large-scale collection regime comprised 178 *T. viridipunctatus*, 87 *T. luyeanus*, 99 *T. formosanus*, and 38 *T. hsuehshanensis* (Table S2). To approach the distribution boundary between *T. viridipunctatus* and *T. luyeanus*, a comparatively intense collection was conducted across the Qingshui Cliff, in specific localities (8–15, Fig. 1C). Two *T. wolteri* collected from eastern China (Fig. 1A), as well as another complete mitochondrial genome sequence (GenBank Accession No. JX181764, Yu and Ji, 2013), were also included in this study.

Mitochondrial DNA sequencing of three genes from the large-scale sampling led us to focus on a narrow contact zone along the Liwu River, a montane river that flows in an eastward direction (Fig. 1D). In non-raining seasons, the major water body of the river is less than 15 m and is sometimes shallow enough to wade across. To detect the fine-scale genetic differentiation across this stream, another intense fine-scale collection was conducted along the river banks from 2004 to 2005. Seven (N1–N7) and eight (S1–S8) sites were chosen from the northern and southern banks of the stream (Fig. 1D), respectively, yielding a total of 263 lizards consisting of 125 northern and 138 southern individuals, respectively. The collection sites of all of the specimens were precisely located using a handheld GPS device (Oregon 550t, GARMIN Ltd., Taiwan). To represent the spatial distribution of possible hybrids, samples from this contact zone were aligned from north to south according to their distance from the stream in the following analyses.

Outgroup included 25 individuals from 11 other *Takydromus* (Table S3), and this study covered a total of 16 species among the 22 currently recognised *Takydromus* spp. Sequences of three closely related lacertids (Fu, 2000) were downloaded from GenBank and were used to root the phylogenetic trees. Genomic DNA of all these specimens was isolated using the DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA) following the manufacturer's instructions and stored at −20 °C.

2.2. Mitochondrial DNA sequencing

Mitochondrial DNA sequencing was applied to all the *Takydromus* individuals in the large-scale sampling. Partial cytochrome *b* (cyt*b*, 702 base pairs after alignment), partial cytochrome *c* oxidase subunit I (COI, 789 bp) and full-length 12S rRNA (1014 bp) were amplified using the following primers designed in this study: CYTB-L: 5′-CGAGAYGTYCAAYAYGGMTGRYTYATYCG-3′; CYTB-H: 5′-ACAAATAAGGTTTGAGAGAGTGGGCG-3′; COI-L: 5′-GCTGGTACYGGCTGAACTGTCTACCC-3′; COI-H: 5′-CARTGNACRAATCCRCCCATRATTGC-3′; 12S-L: 5′-AGTCTGCTCAAAAAGATTAATGTTAA-3′; 12S-H: 5′-TCTTGGTCTGAAACCTCAGTTACCTA-3′. Reactions were conducted in a 20-μl reaction volume containing 1× PCR buffer (10 mM Tris–HCl, pH 9.0; 50 mM KCl, 0.01% (w/v) gelatine and 0.1% Triton X-100), 0.8 U of Taq DNA polymerase (Amersham Bioscience, New Jersey, USA), 0.5 mM dNTP, 0.2 mM each primer and 50 ng of template DNA. The PCR conditions consisted of denaturation at 94 °C for 3 min, followed by 35 cycles of 94 °C for 30 s, 52 °C for 40 s and 72 °C for 90 s, with a final extension at 72 °C for 10 min using an iCycler Thermal Cycler (Bio-Rad). PCR products were purified with the PCR Product Pre-Sequencing Kit (USB Corp., Cleveland, OH, USA) and subsequently used as the template for direct DNA sequencing reactions with the DYEnamic ETDye Terminator Cycle Sequencing Kit (Amersham Pharmacia Biotech). The primers used for PCR were also used for the sequencing reactions. Sequencing products were run on a MegaBACE 1000 automated DNA sequencer (Amersham Bioscience, New Jersey, USA). The sequences were determined in both directions, and the original signals were proofread using SEQUENCHER 4.7 software (Gene Codes Corp., MI, USA). Sequences were aligned by SEQUENCHER 4.7 software with the default settings. All the haplotypes were submitted to GenBank (AY248482–AY248604, KM487140–KM487188).

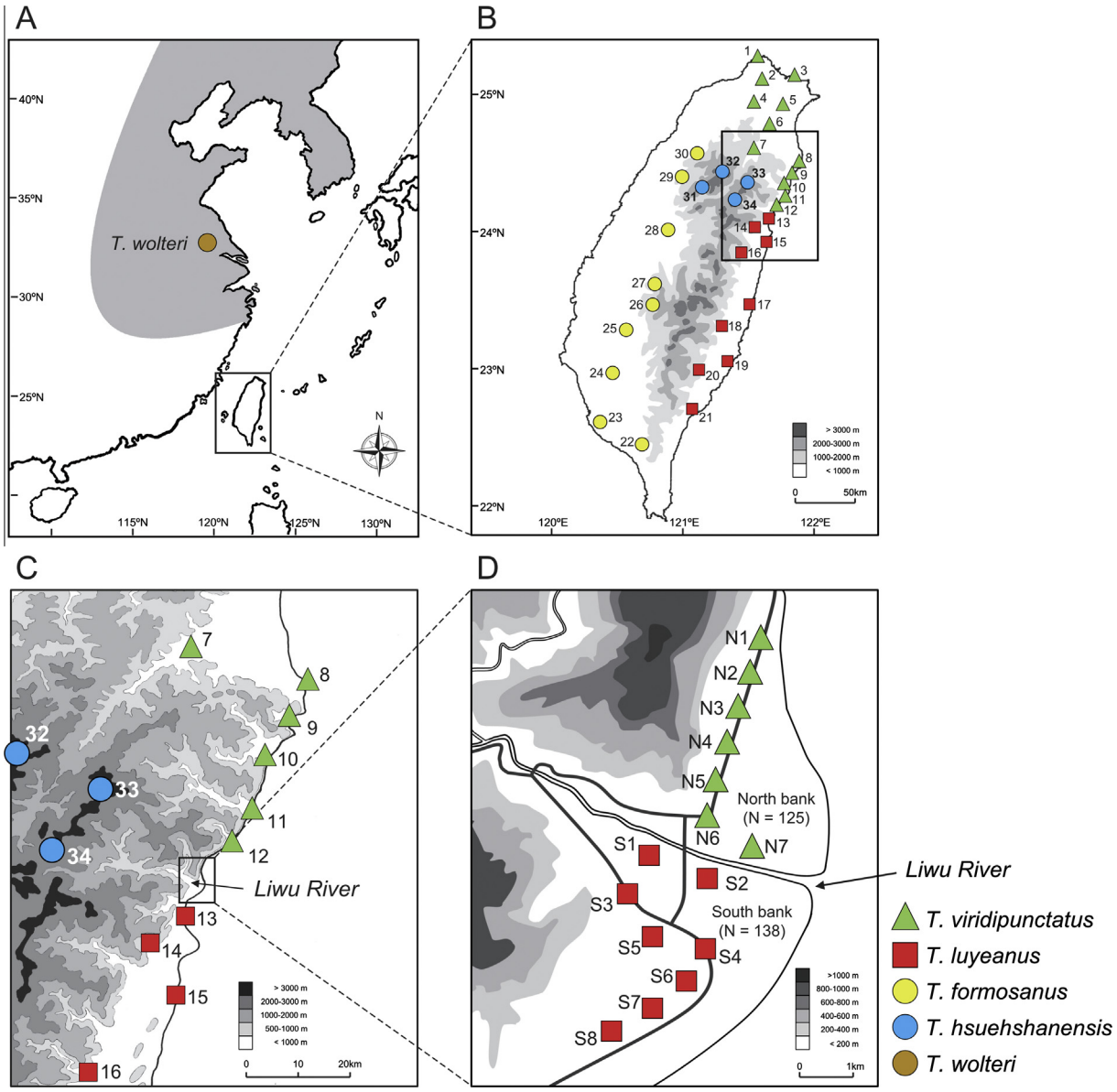

Fig. 1. (A) Sample locality and potential distribution range of *Takydromus wolteri*; (B) the 34 sample localities of the other four members of *T. formosanus* species complex for mitochondrial DNA sequences (populations 1–34, $N = 402$); (C) an extra intense sampling across the boundary between *T. viridipunctatus* and *T. luyeanus* (populations 7–16); and (D) the 15 fine-scale sampling sites across the contact zone for mitochondrial/microsatellite genotyping ($N = 263$). In the following analyses, the 263 individuals were rearranged according to the direct distance from the stream (as located by a GPS machine). Green triangles: *T. viridipunctatus*; red squares: *T. luyeanus*; yellow circles: *T. formosanus*; blue circles: *T. hsuehshanensis*; and orange circle: *T. wolteri*. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2.3. Genetic diversity and population dynamics

The genetic diversity of each population was evaluated with respect to haplotype diversity (H) and nucleotide diversity (pi) calculated using DnaSP 5.10.1 software (Librado and Rozas, 2009). Owing to the low intraspecific variation, 12S rRNA sequences were not included in these analyses. The McDonald–Kreitman test (McDonald and Kreitman, 1991) was applied to exclude the influence of positive/negative selections of cytochrome *b* and COI genes among this species complex (*P*-value = 0.5134, neutrality not rejected).

2.4. Phylogenetic tree reconstruction

Three mitochondrial genes from a total of 433 individuals were sequenced and aligned. All three genes were concatenated in a single matrix for subsequent analyses, yielding a total length of 2505 base pairs after alignments. PartitionFinder 1.0.1 software (Lanfear et al., 2012) was used to determine the best substitution model and partitioning scheme based on Bayesian information criterion (BIC) scores. The results indicated a scheme with four partitions: 12S rRNA + 1st position of cyt*b*, the 2nd position of cyt*b* and COI, the 3rd position of cyt*b* and COI, and the 1st position of COI. The preferred evolutionary model for all four partitions was GTR + I + G.

The best partitioning scheme was used for maximum likelihood (ML) and Bayesian phylogeny inferences. The ML analysis was conducted with RAxML 7.3.2 software (Stamatakis, 2006). The best ML tree was selected from 200 iterations, each starting with distinct randomised parsimony trees. Clade support was examined by non-parametric bootstrap analyses (100 replicates) summarised with 50% majority rule consensus trees. Bayesian inference analyses were conducted with MrBayes 3.2.2 (Ronquist et al., 2012). The combined data matrix was partitioned, and models were assigned as suggested by PartitionFinder. Two independent runs of 5×10^7

generations with 8 MCMC (Markov Chain Monte Carlo) chains each were conducted simultaneously, starting from random trees and resampling each tree every 1000 generations. The standard deviation of split frequencies between runs (<0.01) and the effective sample size (ESS) as measured by the TRACER 1.5 software (Rambaut and Drummond, 2009) were monitored to ensure stationarity, convergence and correct mixing of the chains and to determine the correct number of generations to discard as a burn-in for the analyses (first 20%). Converged MrBayes runs were combined after the exclusion of burn-in, and a majority rule consensus tree was created with nodal confidence assessed by posterior probabilities.

The dataset with all individuals contained many repetitive sequences from the same species, which were not necessary for the following analyses. Hence, we produced a second dataset with a reduced sample size of 80 OTUs to (1) represent a better resolution among ingroup and outgroup species, (2) conduct divergence time estimation, and (3) conduct ancestral range reconstruction. Identical algorithms, including substitution model and partitioning scheme selection (PartitionFinder 1.0.1), ML analysis (RAxML 7.3.2), and Bayesian posterior probabilities (MrBayes 3.2), were conducted. The preferred evolutionary model was the same as the entire dataset, with four partitions (see above) and the GTR + I + G model. For this dataset, the best ML tree was selected from 1000 iterations, while clade support by bootstrap analyses was increased to 2000 replicates. We also performed longer MCMC chains in the Bayesian posterior probability analyses with 5×10^8 generations (10% burn-in).

2.5. Divergence time estimation and ancestral range reconstruction

The divergence times among members of the *T. formosanus* complex were estimated by employing the BEAST 1.7.5 software (Drummond et al., 2012). The mean substitution rate was given as 0.00625/MY/bp, as proposed in Lin et al. (2002). An uncorrelated lognormal clock model, the GTR substitution model, and the Yule speciation process were used in the estimation. MCMC analyses were run for a total of 100 million generations, with sampling every 5000 steps and removal of the first 10% steps as burn-in. The means and 95% confidential intervals of divergence time calculation were labelled on the maximum clade credibility (MCC) tree.

To infer the range and speciation events of members within the *T. formosanus* species complex, we performed a Bayesian approach dispersal-vicariance analysis (Bayes-DIVA) (Ronquist, 1997) implemented in RASP 2.1a software (Yu et al., 2010), which statistically evaluates the alternative ancestral ranges at nodes based on a set of trees (Nylander et al., 2008; Harris and Xiang, 2009). We delimited three areas based on the distribution of related taxa and the presence of marine barriers: Taiwan (T), mainland East Asia (E) and the Ryukyus (R). Ancestral areas were reconstructed on 20,000 randomly chosen post burn-in trees of the BEAST analysis. The maximum number of ancestral areas at each node was set to three. Relative frequencies of ancestral areas reconstructed for the nodes were represented as colour pie charts labelled on the same MCC tree from the BEAST analysis, and ascertained dispersal and vicariance events were labelled as red circles with solid and dotted lines, respectively.

2.6. Microsatellite genotyping in the contact zone

Microsatellite genotyping was applied to the 263 samples collected from the contact zone. To compare these samples to the pure lines, 75 *T. viridipunctatus* from localities No. 8–12 and 44 *T. luyeanus* from localities No. 13–17 were included in microsatellite genotyping. Individual genotypes were assessed using 13 microsatellite loci published by Lin et al. (2006). PCR products were electrophoresed in a MegaBASETM 1000 autosequencer (Amersham Bioscience, New Jersey, USA) with the size marker ET-400 (Amersham Bioscience, New Jersey, USA). Alleles were scored manually with the aid of GENETIC PROFILER 2.2 software (Amersham Bioscience, New Jersey, USA).

The number of alleles (N_A), observed (H_O) and expected (H_E) heterozygosities for each locus were calculated using POPGENE 1.31 software (Yeh et al., 1997). GENEPOP 4.0 online version software (http://genepop.curtin.edu.au/) was used to evaluate significant deviations from the Hardy–Weinberg Equilibrium (HWE). Micro-checker software (Van Oosterhout et al., 2004) was used to check the presence of null alleles. Finally, input files for the following analyses were created using PGDSpider 2.0 software (Lischer and Excoffier, 2012).

2.7. Mitochondrial DNA typing in the contact zone

To determine the genetic assignment of mitochondrial sequences from each lizard in the contact zone along the Liwu River, two sets of species-specific primers were designed from sequences with fixed inter-specific divergence on the COI gene: ViL (5′-TTGTAGAGTGAGTATGGGGG-3′) and ViH (5′-TTGTTTTGAT AAATGAGTGA-3′ are specific for *T. viridipunctatus*; LuL (5′-TCGTA GAGTGGGTATGAGGC-3′) and LuH (5′-TTGCTTTGATAGGTGAATA T-3′) are specific for *T. luyeanus*. Each sample along the contact zone (with a total of 263 individuals) was PCR amplified twice using the ViL–ViH pair and twice using the LuL–LuH pair. A total of 257 of the 263 samples (97.7%) represented positive bands from only one of the primer pairs, suggesting a high diagnostic rate for this method. The remaining six samples, which did not show positive bands from either primer pair, were amplified by the universal primers (COI-L vs. COI-H) and were identified by the DNA sequencing process, as previously described.

2.8. Detection of hybridisation in the contact zone

Two methods were used to detect hybrid individuals in the contact zone using the microsatellite dataset from the 263 individuals. The population structure in this region was determined using the Bayesian model-based clustering software STRUCTURE 2.3.4 (Pritchard et al., 2000; Hubisz et al., 2009). The number of assumed genetic clusters (K) was set from 1 to 8, and 15 runs were performed for each K with 1,000,000 MCMC iterations (the first 200,000 iterations were discarded as burn-in). For each dataset, 15 runs were conducted to assess the degree of variation of the likelihood for each K. The best K value was obtained based on the ΔK (delta K) that was estimated by the Evanno method (Evanno et al., 2005) using Structure Harvester software (Earl and von Holdt, 2012). However, the result of $K = 2$ was retained as a reference, according to the taxonomic basis of two valid species.

Q-values were calculated with 0.95 posterior confidence regions (CR) describing the posterior probability of an individual's genotype belonging to each identified group. Hybrids can then be inferred when an individual is an intermediate of some degree between two clusters in terms of the q-value, i.e., a first-generation hybrid should have a q-value of 0.5. Individuals whose 95% CR included 0.50 were identified as intermediates (Schrey et al., 2007; Boley and Edward, 2011).

A Discriminant Analysis of Principal Components (DAPC) (Jombart et al., 2010), available in the R package adegenet (Jombart, 2008), was used to investigate the structure of populations using genetic data. Population labels were given as the prior cluster information in DAPC. The first 80 principal components (PCs), which explained 80% of the total microsatellite genetic variability, were retained in the following procedure. The first two DA axes were sufficient to summarise the genetic variation among populations and

produced a scatter plot that provided an illustration of microsatellite genetic structure. Individuals with membership posterior probabilities of their own species lower than 0.875 were determined to be potential hybrids in the DAPC assignment test.

2.9. Gene flow estimation

Two different methods were used to evaluate the migration pattern between the two populations, which showed the short-term and long-term migration rates, respectively, and identified the level and extent of genetic introgression beside the contact zone. The program BayesAss 3.0 was used to estimate recent migration rates within a comparatively recent time span (within a few generations, Wilson and Rannala, 2003). BayesAss uses a Bayesian framework to infer migrations with multilocus genotyping data (Wilson and Rannala, 2003). The calculation was conducted with 5×10^7 generations and sampling every 1000 steps, and the first 5×10^6 generations were discarded as burn-in. The result from BayesAss was summarised by Tracer 1.5 software (Rambaut and Drummond, 2009).

Migrate-N 3.6 was applied to assess long-term migration rates (last 4N generations) using the likelihood method (Beerli and Felsenstein, 2001; Beerli and Palczewski, 2010). The analysis was conducted with 10 short chains with an increment of 100 and a sampling of 5000 steps and 2 long chains with an increment of 100 and a sampling of 50,000 steps. The first 10,000 steps were discarded as burn-in for each chain in the analysis. Because of the high polymorphism level of the microsatellite loci, the Brownian microsatellite model was used in the Migrate-N analysis. We further tested different models in the Migration-N analysis using likelihood ratio tests. First, we tested whether the long-term migration rate between the two species were significantly different from zero. Second, we tested whether the migration rates were equal in two opposite migration directions.

3. Results

3.1. Phylogeny of the Takydromus formosanus species complex

A phylogenetic tree containing all samples of the *T. formosanus* species complex (433 OTUs) is represented by the ML tree shown in Fig. 2. The phylogeny constructed by Bayesian analyses resulted in identical tree topology, with the posterior probability values labelled on their corresponding nodes. According to this result, all specimens were correctly assigned to their own genetic clade with no exception, showing congruence with genetics, morphology and geographic distribution. This result indicated that all the individuals were identified with no ambiguity, with non-sympatric occurrence among the four species under this large-scale sampling regime (Fig. 1).

The phylogeny of the entire genus was represented by the ML tree created from the reduced OTUs, as represented in Fig. 3. *Takydromus wolteri* formed a highly supported clade with *T. formosanus*, while *T. viridipunctatus* and *T. luyeanus* were clustered as sister species. These four species were further grouped with the alpine *T. hsuehshanensis*, leading to a five-species clade with four species endemic to Taiwan. The sister clade of the *T. formosanus* species complex is the *T. septentrionalis* species complex (Fig. 3), comprised of *T. septentrionalis* (mid-southern China), *T. stejnegeri* (endemic to Taiwan), and *T. toyamai* (endemic to Miyako Island of the southern Ryukyus).

3.2. Divergence times and ancestral ranges

Mitochondrial sequence polymorphisms of each population and clade of the *T. formosanus* complex are listed in Table S1 and summarised in Table S2. Interspecific divergence of the three genes are averaged and listed in Table S4. Members within the complex showed prominent inter-specific divergences (*p*-distance) ranging between 0.0639 (*T. formosanus* vs. *T. wolteri*) and 0.0861 (*T. hsuehshanensis* vs. *T. formosanus*), which were much higher than the intra-specific variation (0.0024–0.0132).

The mean and 95% confidence interval of each speciation event (in MYA) were labelled on the maximum clade credibility (MCC) tree as a chronogram shown in Fig. 4. The *T. formosanus* complex separated from the *T. septentrionalis* complex at 3.56 MYA (95% confidence intervals: 1.83–5.59 MYA). Within the species complex, *T. hsuehshanensis* was recovered as the basal species, with a 3.19 MY (1.65–5.10 MYA) divergence history. At 2.82 MYA (1.46–4.64 MYA), the remaining species further separated into two clades. Speciation between *T. viridipunctatus* and *T. luyeanus* occurred at 2.11 MYA (0.98–3.50 MYA), and speciation between *T. wolteri* and *T. formosanus* occurred at 2.07 MYA (0.88–3.46 MYA).

DIVA analysis using RASP confirmed six speciation events (Fig. 4), including three dispersals (nodes 1, 2, and 4, denoted as red solid circles) and 3 vicariances (nodes 5, 7, and 8, denoted as red dashed circles). Two events were directly relevant to our focal group: a dispersal led to a range expansion of this complex from Taiwan to mainland China (node 4) and a vicariance led to speciation between *T. formosanus* and *T. wolteri* (node 5). We noticed that some other speciation events within the complex were not highlighted by DIVA analysis because these events did not involve distributional range change (e.g., nodes 3 and 6). These results indicated that the speciation events within the complex did not experience oversea vicariance or dispersal, providing strong evidence for within-island speciation.

3.3. Hybrid assignment in the contact zone from STRUCTURE

A total of 382 samples, 263 from the contact zone (Fig. 1D) and 119 from pure lines, were successfully genotyped for their 13 microsatellite loci (Table S5). Of the 156 tests for conformity with the Hardy–Weinberg equilibrium (12 populations at 13 loci), three were significant after a Bonferroni correction for multiple tests (critical value = 0.00032). The result of Micro-checker analysis indicated the presence of null alleles in locus TF163 in six *T. viridipunctatus* populations in our study. Because of a high level of polymorphism and low probability of homozygosity, we treated one of TF163 homozygous alleles as missing data for the following analyses.

The existence of three genetic groups ($K = 3$) was determined based on ΔK. The entire sample could be divided into three major groups: a pure-line *T. viridipunctatus*, the population of *T. viridipunctatus* on the northern bank of Liwu River, and all of the *T. luyeanus* individuals south of the stream (Fig. 5A). This result is congruent with the two-group designation (based on the two-species taxonomy), which demonstrates a consistent distinct differentiation located precisely at the stream (Fig. 5B).

Under the definition of hybrids (0.95 posterior confidence regions of *Q*-values cover 0.5), a total of 26 samples were determined to be hybrids when $K = 3$, including 8 from the northern bank and 18 from the southern bank (Table 1). The resultant ratio of hybrids within the contact zone was 6.4% (8/125) on the northern bank, 13.0% (18/138) on the southern bank and 9.9% (26/263) in total (Table 1). When $K = 2$, the number of detected hybrids is 3.2% (4/125) on the northern bank, 8.0% (11/138) on the southern bank and 5.7% (15/263) in total (Table 1).

Genetic assignment based on maternal inheritance also demonstrates the distinct differentiation at the Liwu River (Fig. 5C). Among the 263 individuals evaluated in the contact zone, all but one individual were correctly assigned to the predicted local lineages, i.e., 125 individual from the northern bank belonged to *T. viridipunctatus*, and 138 from the southern bank belonged to

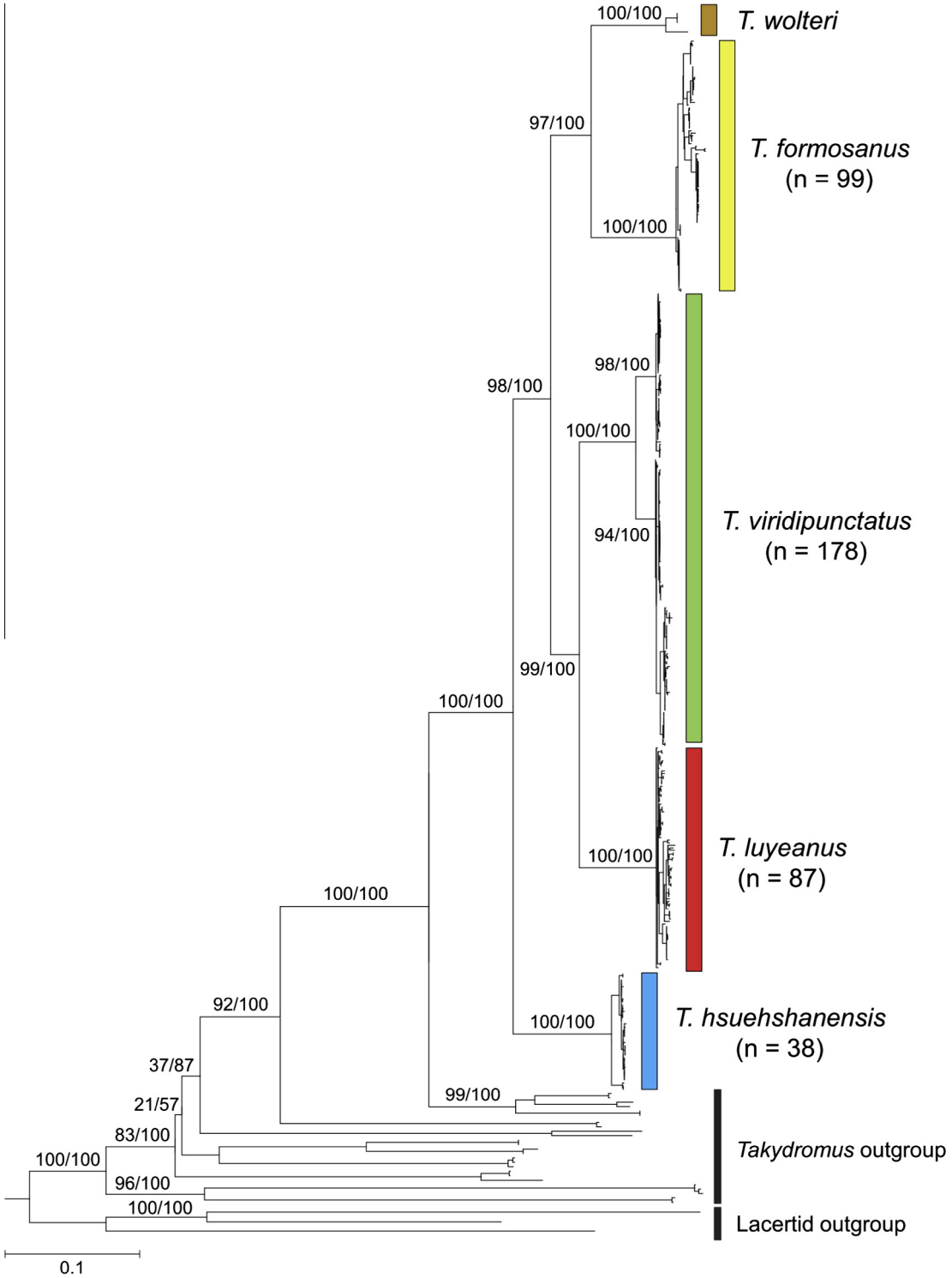

Fig. 2. Maximum likelihood (ML) tree of the *Takydromus formosanus* species complex from concatenated sequences of the mitochondrial cytochrome *b*, COI, and 12S rRNA genes (2505 base pairs). This dataset comprised 433 sequences including 3 *T. wolteri*, 178 *T. viridipunctatus*, 87 *T. luyeanus*, 99 *T. formosanus*, 38 *T. hsuehshanensis*, 25 other *Takydromus* species, and 3 lacertid outgroups. Statistical support of each node was represented by a 100 ML bootstrap replicates and Bayesian posterior probabilities. This phylogeny helped to confirm the genetic and geographic boundaries between these parapatric taxa; no ambiguous individual was found in this sampling regime.

T. luyeanus. The only exception occurred on the southern bank, where a hatchling lizard carried a *T. viridipunctatus* mitochondrial haplotype and seemed to be an F1 hybrid based on microsatellite genotyping (the small arrow in Fig. 5C). Therefore, a higher proportion of genetic introgression across the river stemmed from male introgression instead of female introgression. The sampling did not detect an intruder that had crossed the river (i.e., a genetically pure-line individual found on the other side stream from their original distribution).

3.4. Hybrid assignment in the contact zone based on DAPC

The result of DAPC from microsatellites is shown in Fig. 6. Pure-line *T. viridipunctatus* (populations 8–12, in dark green)

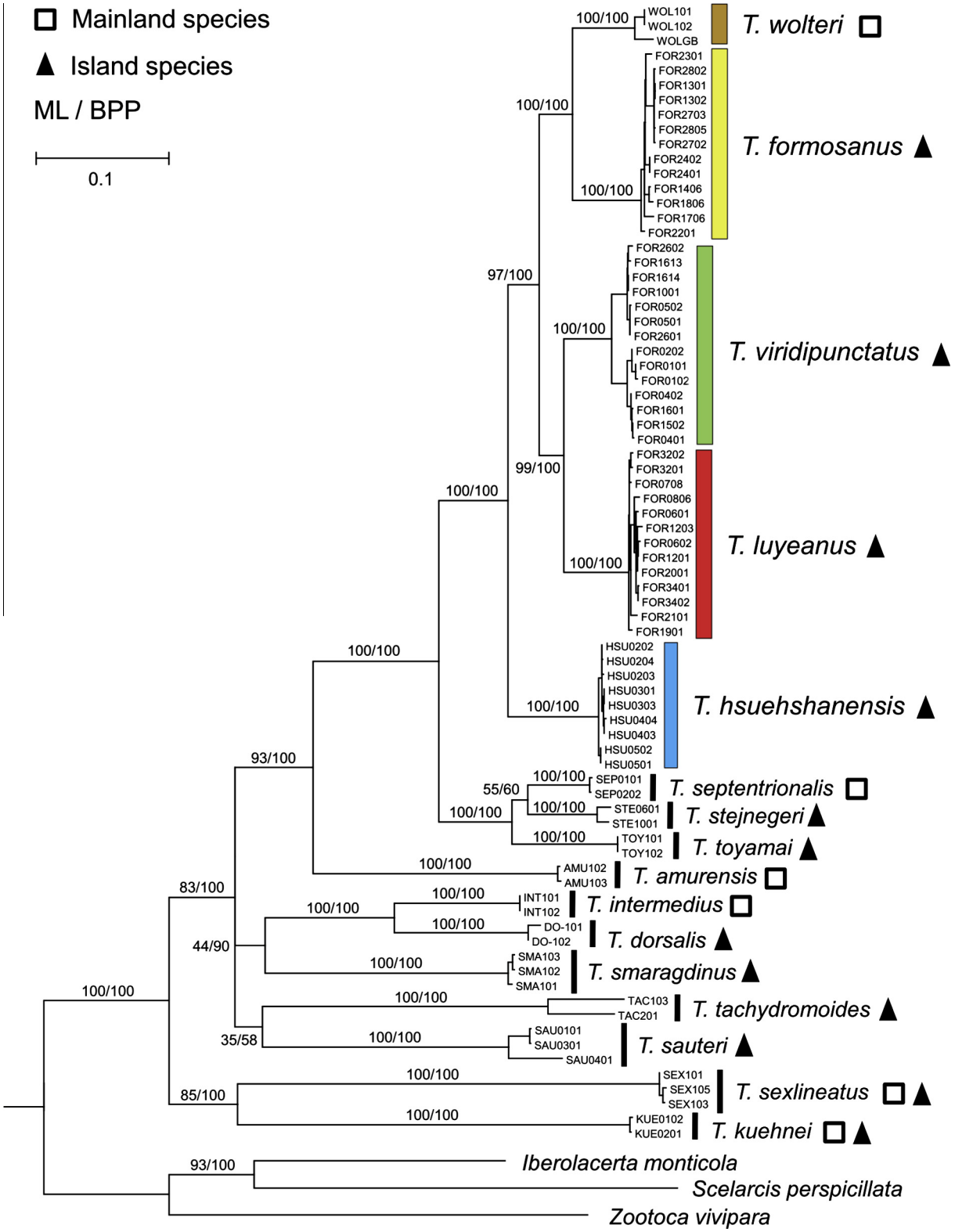

Fig. 3. Maximum likelihood (ML) tree of the *Takydromus formosanus* species complex from concatenated sequences of the mitochondrial cytochrome *b*, COI, and 12S rRNA genes (2505 base pairs). Statistical support of each node was represented by a 2000 ML bootstrap replicates and Bayesian posterior probabilities. Insular and mainland species are denoted by triangles and rectangles, respectively. This phylogeny provided evidence for (1) within-island speciation of *T. formosanus* species complex and (2) the unusual backward dispersal route from island to mainland, which occurred in *T. wolteri*, *T. septentrionalis*, and *T. intermedius*.

individuals displayed contiguous distribution on the DAPC scatter plot similar to their actual geographic distribution. These five pure-line populations were then connected to lizards from the northern bank of the Liwu River (in light green). Compared to *T. viridipunctatus*, *T. luyeanus* represented a lower population differentiation among the southern bank population (light red) and other pure-line populations (dark red, populations 13–17). This result, congruent with the results from STRUCTURE and mtDNA

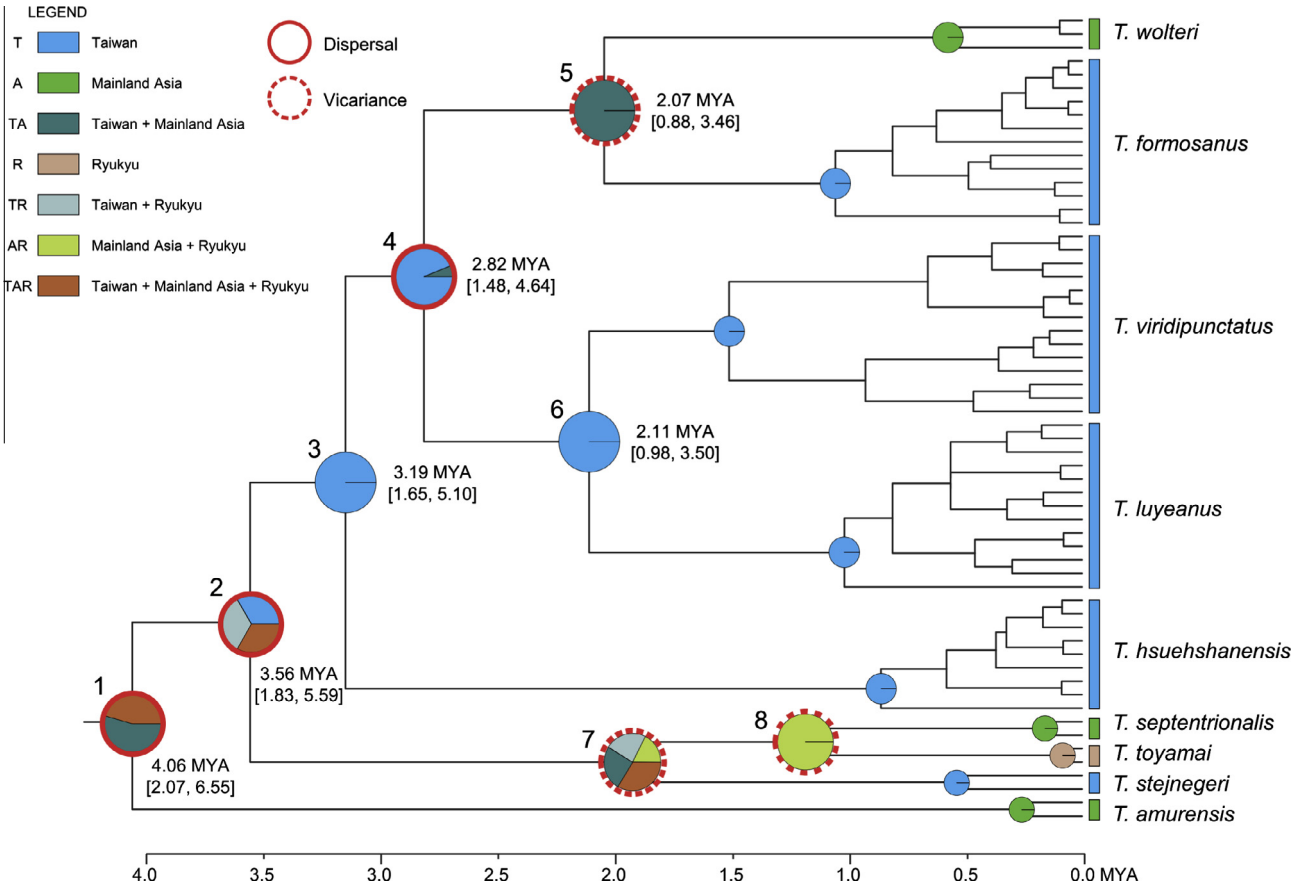

Fig. 4. Divergence time of *Takydromus* lizards estimated using BEAST 1.7.5 and ancestral range reconstruction using Bayesian approach dispersal-vicariance analysis (Bayes-DIVA) implemented in RASP 2.1a. Divergence times are shown beside the nodes with 95% highest posterior densities in the brackets. Six distributional events, including three dispersals (nodes 1, 2, and 4, denoted as red solid circles) and three vicariances (nodes 5, 7, and 8, denoted as red dashed circles), were confirmed by Bayes-DIVA. Except for *T. wolteri*, other speciation events of the *T. formosanus* species complex did not involve distributional change, which provided robust evidence of within-island speciation.

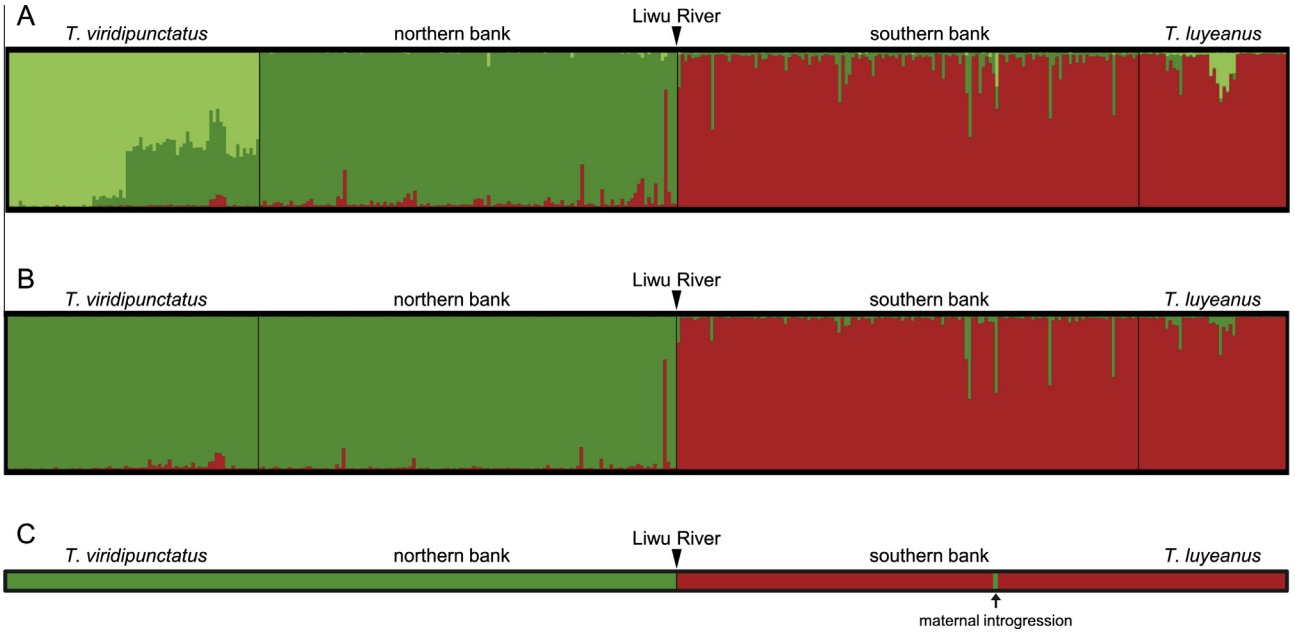

Fig. 5. Population differentiation among pure-line *Takydromus viridipunctatus* ($N = 75$), pure-line *T. luyeanus* ($N = 44$), and samples collected from their contact zone (125 from the northern and 138 from the southern bank). (A) The population genetic structure as determined using STRUCTURE, with the optimal grouping number $K = 3$; (B) the population genetic structure when K was designated as 2, based on our taxonomic knowledge; (C) the genotyping of each individual based on mitochondrial sequences.

Table 1
Number and ratio of hybrids in the contact zone between *T. viridipunctatus* (northern bank) and *T. luyeanus* (southern bank) assigned by STRUCTURE ($K = 3$ and $K = 2$) and DAPC based on microsatellite data.

Region	*n*	STRUCTURE ($K = 3$)	STRUCTURE ($K = 2$)	DAPC
Northern bank	125	8 (6.4%)	4 (3.2%)	3 (2.4%)
Southern bank	138	18 (13.0%)	11 (8.0%)	3 (2.2%)
Total	263	26 (9.9%)	15 (5.7%)	6 (2.3%)

genotyping (Fig. 5), indicated that lizards from either side of the bank represented high genetic differentiation and seldom crossed the border.

Among the 263 individuals collected from the contact zone, DAPC assignment indicated 6 misclassified individuals (membership posterior probabilities < 0.875): 3 from the northern and 3 from the southern banks. Hybrid ratio estimates were slightly lower in DAPC than in STRUCTURE: 2.4% (3/125) on the northern bank, 2.2% (3/138) on the southern bank and 2.3% (6/263) in total (Table 1).

3.5. Migration in the contact zone

Although a higher proportion of hybrids occurred on the southern bank, the recent migration rate estimated using BayesAss software was not significantly different between the two directions (Table 2). The median value of the recent migration rate (m = proportion of population) was 0.0043 from north to south, which was slightly lower than the value of 0.0046 from south to north. However, the long-term migration rate ($M = m/\mu$; migration rate scaled by the mutation rate) estimated using Migrate-N was 4.2372 from north to south and 3.6633 from south to north (Table 2). The effective population size ($\Theta = 4\,N\mu$; population size scaled by mutation rate) estimated by Migrate-N was 1.0886 and 1.2058 on the northern and southern banks, respectively (Table 2). With an approximate likelihood ratio test, the null hypothesis of zero migration rates between two populations was rejected (P-value < 0.0001). The model of symmetric migrations between two populations (H_0: $M_{V\to L} = M_{V\to L}$) could not be rejected in the likelihood ratio test (P-value = 0.118). These results indicated that the two species experienced historical gene flow, which might have gradually ceased in recent years.

4. Discussion

4.1. Geographic boundary and fine-scale differentiation

Sibling species with parapatric distribution are always an interesting issue in evolutionary biology (Abbott et al., 2013; Hoskin and Higgie, 2013). The most distinguished discovery of this study is the distinct spatial distribution of *T. viridipunctatus* and *T. luyeanus*, which are presently separated by a narrow stream. In non-raining periods, the major water body of the stream is less than 15 m and is sometimes shallow enough to wade across. Although many well received studies have identified rivers as biogeographic barriers, almost all studies either discuss very large geographic scales, such as the Amazon River (Peres et al., 1996; Hayes and Sewlal, 2004), or address an intraspecific level (Heulin et al., 2011). A species boundary maintained by a narrow stream on such a small scale is seldom observed in terrestrial vertebrates, and this situation has never been reported in East Asia. The case of these two sister lizard species provides a valuable chance to study the process of speciation in a narrow contact zone.

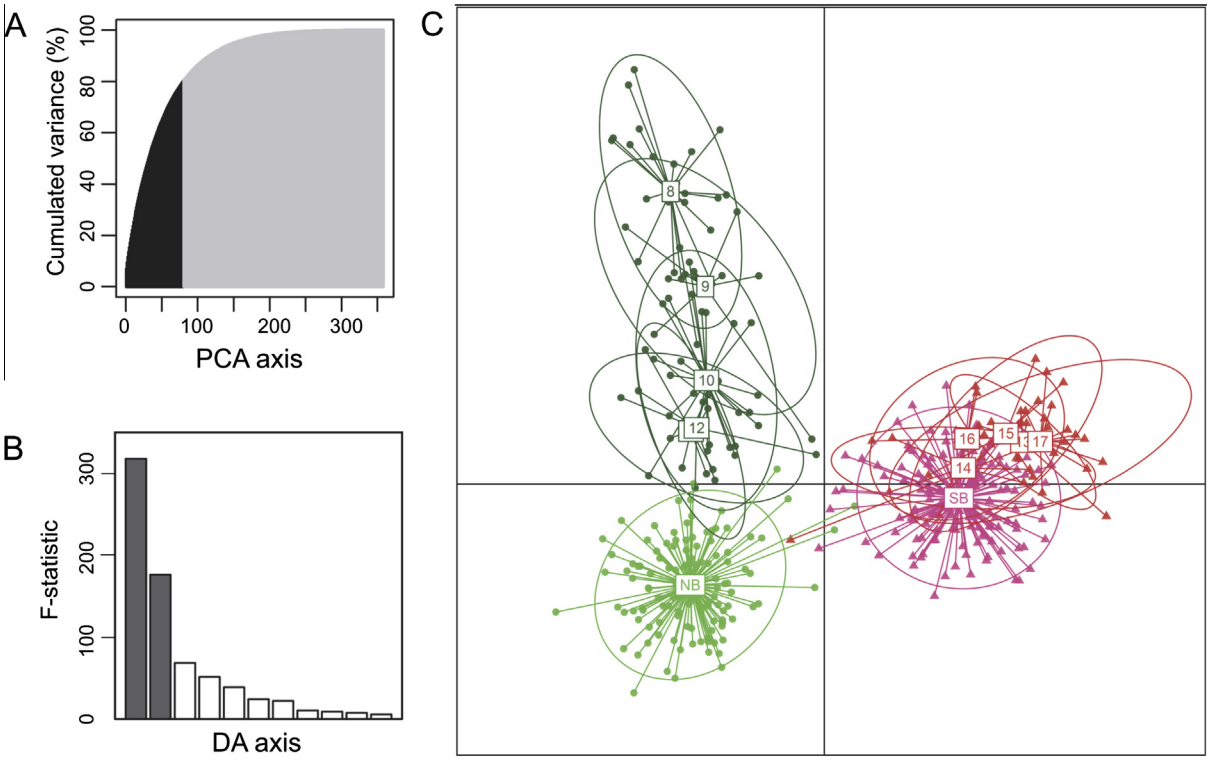

Fig. 6. (A) Variance explained by PCA in DAPC. The first 80 PCA components were retained (in black), which explained 80% of total microsatellite genetic variability. (B) Bar plot of eigenvalues for the discriminant analysis with *F*-statistic values (= ratio of between-group and within-group variances). (C) Scatter plots of pure-line *Takydromus viridipunctatus* (dark green circles, $N = 75$), pure-line *T. luyeanus* (dark red triangles, $N = 44$), northern bank of the contact zone (light green, $N = 125$), and southern bank of the contact zone (light red, $N = 138$) on the first two axes of DAPC. Population numbers are labelled in the centre of the dispersion, while the large open circles indicate the 90% inertia ellipses for each population. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 2
Short term and long term migration estimates between northern and southern bank populations using BayesAss (top) and Migrate-N (bottom).

Short term migration	Highest posterior density	95% HPD lower	95% HPD upper
$m_{V\to L}$	0.0043498	0.0000003	0.0132620
$m_{L\to V}$	0.0046390	0.0000006	0.0136770
Long term migration	Maximum likelihood estimates	95% HPD lower	95% HPD upper
$M_{V\to L}$	4.2372	3.8699	4.6363
$M_{L\to V}$	3.6633	3.3255	4.0322
Θ_V	1.0886	1.0336	1.1589
Θ_L	1.2058	1.1427	1.279
$Nm_{V\to L}$	1.2773		
$Nm_{L\to V}$	0.9970		

Abbreviations: V: *T. viridipunctatus*; L: *T. luyeanus*; *m*: migration rate; *M*: scaled migration rate m/μ; Θ: effective population size $4N_em$; Nm: effective migrants per generation.

The geographic position of the Liwu River is located along the Qing-shui Cliff, the steepest and easternmost corner of the Central Mountain Range. This mountain range has long been regarded as the most prominent geographic barrier in Taiwan (Tzeng, 1986; Wang et al., 2004; Lin et al., 2008, 2012). During 3.0–2.8 MYA, a severe collapse between Eurasian Plate and Philippine Plate uplifted this region, thus creating an extremely steep cliff zone beside the ocean (Sibuet and Hsu, 2004). Consistent results from other phylogeographic studies indicated that this region disrupted the gene flow between the northern and southern populations for amphibians, reptiles, and freshwater fishes (Wang et al., 2004; Jang-Liaw et al., 2008; Lin et al., 2012; and also see the review in Lin et al., 2012). Importantly, the timing of this geological event precisely fits the divergence time between *T. viridipunctatus* and *T. luyeanus* estimated by BEAST. Although the role of this mountain range is prominent, fine-scale studies across this region were conducted only for a tree frog (Lin et al., 2012) and in the present study. Comparison of these two species showed an amazingly precise congruency with their common geographic barrier.

4.2. Genetic boundary and male-biased introgression

A total of 26 individuals were determined to be hybrids in STRUCTURE ($K = 3$), yielding a ratio of 9.9% hybrids (Table 1). Determination of hybrids based on DAPC and STRUCTURE, when *K* was designated as 2, revealed a lower ratio: 2.3% (6 individuals) from the northern bank and 5.7% (15 individuals) from the southern bank, respectively. No intruder (pure-line individual occurring in the opposite site) was directly detected in our sampling, indicating a low probability for the lizards to cross the stream, which would result in the extremely low levels of recent gene flow indicated in Table 2 ($m = 0.0043$ from *T. viridipunctatus* to *T. luyeanus*; $m = 0.0046$ from *T. luyeanus* to *T. viridipunctatus*). This situation is sometimes explained as the result of secondary contact (Hewitt, 1988; Coyne and Orr, 2004). Considering the existence of genetic differentiation between the northern bank population and the other pure-line *T. viridipunctatus* individuals (Fig. 5A), the age for this contact history must not be recent.

The long-term maintenance of the two sister taxa by only the narrow stream seems to be a paradox: these lizards inhabit open grassland of an early successional stage and form extremely large populations along the river banks. During the summer, frequent rainstorms and typhoons can easily transport individuals across the water. The stream does not seem to completely stop their movements. Under these conditions, it is not clear how this border is maintained. The most probable explanation is prezygotic isolation mediated by the differentiation of the lizards' nuptial colouration, represented by the green spots in *T. viridipunctatus* and the yellow spots in *T. luyeanus*. According to our recent experiments (S.-M. Lin et al., unpublished data), the nuptial colour can be strengthened when testosterone is provided to the males. Females are attracted to those males with the more attractive nuptial colouration and may exhibit specific preferences for their own type.

Another notable phenomenon is that the current gene flow across the stream was dominated by male-biased introgression: only one of the hybrids carries a mitochondrial genome from the other side. The male-biased genetic introgression could account for two possibilities: male-biased dispersal or Haldane's rule. A seven year capture-mark-recapture program of *T. viridipunctatus* proved that males have a higher mobility and a larger home range than females, which might facilitate a higher probability of crossing the river (S.-M. Lin et al., unpublished data). However, considering that most Lacertidae with available information belong to the ZW or ZZW sex determination system, females are the sex that carries heterogametic sex chromosomes. Haldane's rule (Haldane, 1922) indicates that a higher rate of mortality and sterility might occur in female hybrids, which not only explains the male-biased genetic introgression but also explains the low level of current gene flow between the two species.

4.3. Within-island speciation with ancient gene flow

Within-island speciation was thoroughly addressed in the West Indies, where *Anolis* lizards form extremely high species diversity due to adaptive radiation (Losos et al., 1998). Malagasy poison frogs (Rabemananjara et al., 2007) and leaf chameleons (Townsend et al., 2009) in Madagascar are other well known cases with high species diversity within a single island. The results of this study represent further evidence of within-island speciation on a medium-sized island. Except for a handful of cases that usually occur in amphibians with low dispersal ability (e.g., Yoshikawa et al., 2012; Tominaga et al., 2013; Blackburn et al., 2013), this study is one of the first few cases that has been robustly verified on East Asian islands.

Until the start of the 21st century, the allopatric speciation model (Mayr, 1942) was the most dominant and widely accepted hypothesis in evolutionary biology (Mayr, 1942; Coyne and Orr, 2004). In most cases, medium to small-sized islands are less likely to harbour a great barrier that can maintain long-term isolation. Therefore, the "strict" allopatric speciation model may not provide a convincing explanation for the formation of *Takydromus* diversity in Taiwan. In recent years, the alternative "parapatric speciation" model (Wu, 2001; Gavrilets, 2003; Coyne and Orr, 2004) predicted that a certain amount of gene flow might persist for a long period before the sister taxa are reproductively isolated. In this study, the null hypothesis of zero migration rates in a large time scale has been rejected (P-value < 0.0001), indicating the existence of historical gene flow between the two species during the process of speciation. The weakness in this study is the unavailability of mutation rates for microsatellite loci, which prevents a comparison

between the results of long-term migration and recent migration. Furthermore, we need a better method of discriminating between historical gene flow and secondary contact because the latter was already detected within the current contact region. Therefore, a sequencing project of multiple nuclear loci from wider samples is needed. We initiated such a program recently, and fruitful results are expected with the assistance of powerful tools for multiple-gene analyses. The evolution of *Takydromus* lizards has undoubtedly provided a novel system to study the formation of insular biodiversity on East Asian islands, a topic that was previously understudied and presents a great potential to be further developed in the near future.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ympev.2015.04.022.

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