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ORIGINAL ARTICLE

Did a Miocene–Pliocene island isolation sequence structure diversification of funnel web spiders in the Taiwan-Ryukyu Archipelago?

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ABSTRACT

Aim We tested the competing hypotheses concerning the relative importance of Pleistocene versus Miocene–Pliocene geological events for the formation of endemism in an Asian archipelago using the *Macrothele taiwanensis* (Hexathelidae) species group.

Location Taiwan-Ryukyu Archipelago.

Methods We estimated phylogenetic trees from cytochrome oxidase I subunit (*COI*) and *16S* rRNA (*16S*) gene regions and employed Bayesian ancestral range reconstructions to investigate previously debated models of lineage diversification in the Taiwan-Ryukyu Archipelago. To evaluate alternative geological timeframes for their importance in shaping the genetic structure of funnel web spiders, we used five time calibration schemes to estimate timing of divergence, infer ancestral distributions, and to reconstruct historical demographic changes in each lineage. We tested taxonomic boundaries with two species delimitation procedures.

Results Our results indicate a north-to-south isolation sequence of the *M. taiwanensis* group: the Amami lineage diverged first, then Yaeyama, and finally the Taiwanese lineages. Divergence time estimation and population demographic change analyses indicate that Pleistocene climate fluctuations minimally impacted the genetic structure of these spiders. Instead, estimated divergence events correspond to Miocene–Pliocene geological events, strongly supporting a much older timeframe for diversification. The results of species delimitation analyses coincide well with morphological differences observed among the island populations, reinforcing inferred species boundaries, and at least three potential cryptic species were statistically detected within Taiwan.

Main conclusions Miocene–Pliocene geological events appear to have contributed disproportionately to diversification in the *M. taiwanensis* species group. The clear association between geographical area, genetic structure and statistical species delimitation strongly supports an interpretation of allopatric speciation. We advocate comparing our results with those derived from additional study organisms with similar life histories to further explore the Miocene–Pliocene diversification hypothesis.

Keywords

allopatric speciation, continental islands, cryptic species, endemism, *Macrothele*, phylogeography, species delimitation

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INTRODUCTION

The biodiversity of continental islands largely is affected by proximity to neighbouring continents and by their historical connections to adjacent mainland via land bridges (Lomolino *et al.*, 2006; Whittaker & Fernández-Palacios, 2007). In contrast to oceanic islands, in which biogeographical processes typically are dominated by dispersal and *in situ* speciation

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(Sanmartín *et al.*, 2008; Gillespie *et al.*, 2012; Brown *et al.*, 2013), vicariance models may better explain the diversification of biota of continental islands (Whittaker & Fernández-Palacios, 2007; Whittaker *et al.*, 2008; but see Sanmartín *et al.*, 2008). Before the application of model-based approaches to hypothesis testing in biogeography, *ad hoc* explanations for distributional patterns usually were necessary; these, of course, have been generally criticized as excessively descriptive (Wen *et al.*, 2013). Frequently, in cases of the study of multiple co-distributed taxa, shared patterns of area relationships are observed, possibly leading to the inference of a general mechanism driving diversification (Ota, 1998; Chiang & Schaal, 2006; Brown *et al.*, 2013; Oaks *et al.*, 2013). Although shared mechanism hypotheses can be proposed to explain observed patterns and they lead to testable predictions, such approaches nonetheless may be limited by a tendency towards possibly overlooking, taxon-specific, alternative explanations. Recently developed model-based approaches (Ronquist & Sanmartín, 2011) have improved the power of hypothesis testing in biogeography. Here, we use model-based approaches to investigate the geographical range evolution in funnel web spiders of the *Macrothele taiwanensis* species group (Hexathelidae), and to evaluate alternative hypotheses for explaining faunal diversification of the Taiwan-Ryukyu Archipelago.

The Taiwan-Ryukyu Archipelago is composed of a string of continental islands along the western edge of the Pacific Ocean. Although initial formation of this island arc may have been facilitated by the opening of the Okinawa Trough in the Miocene (Sibuet *et al.*, 1995; Wang *et al.*, 2014), it is believed that Quaternary glacial cycles have also influenced the biodiversity accumulation in the archipelago via sea level fluctuations and the formation of land bridges with the neighbouring mainland (Ota, 1998). Hence, biotic diversification in the Taiwan-Ryukyu Archipelago has been considered largely the consequence of ephemeral land connections with the Eurasian continent during Pleistocene glaciation cycles (Ota, 1998; Chiang & Schaal, 2006). Due to the NE–SW arrangement of the Ryukyu Arc, only its southern islands were connected to the continent during glacial periods, a geological arrangement that may have resulted in a pattern of northward sequential colonization of mainland-derived biota (Ota, 1998; Chiang & Schaal, 2006). During the most severe glaciations, a peninsula may have formed, extending from the Asian continent, northern Taiwan, to central Ryukyu, that is, Amami and Okinawa island groups (Fig. 1a; Kimura, 2000). This Pleistocene Stepping Stone (PSS) hypothesis, which predicts the existence of ancestral lineages in Taiwan, attributes Pleistocene events, e.g. sea level fluctuations, as the governing geographical processes in shaping phylogeographical patterns in this archipelago (Chiang *et al.*, 2006; Nakamura *et al.*, 2010; Mitsui & Setoguchi, 2012; Muraji *et al.*, 2012). The PSS hypothesis also predicts that all speciation events were relatively recent (i.e. < 2.5 Ma). However, substantial endemism in the Ryukyu Arc (Ota, 1998) cannot be explained exclusively via this hypothesis if speciation processes occurred earlier than 2.5 Ma.

One alternative, but rarely considered, geology-based explanation for shaping Ryukyu biodiversity is the possibility of pre-Pleistocene isolation of the Ryukyu Islands. The dissociation of Ryukyu Islands from the Eurasian continent was strongly associated with the extension of the Okinawa Trough, which can be divided into one Miocene and two Pleistocene phases (Sibuet *et al.*, 1995; Iryu *et al.*, 2006; Wang *et al.*, 2014). The first proto-formation phase of these continental islands occurred after the initial opening of the Okinawa Trough in the late Miocene (Lee *et al.*, 1980; Sibuet *et al.*, 1995; Gungor *et al.*, 2012). This interpretation was supported by stratigraphical evidence including the transition from the early Miocene Yaeyama non-marine deposit to the late Miocene–early Pleistocene Shimajiri marine deposit between the continent and the Ryukyu Arc (Iryu *et al.*, 2006). The reconstruction of the pre-Pleistocene tectonic movements of this island arc is complex and evidence is patchy, but a general agreement among geologists suggests that the initial separation of northern portions of the arc from the continent began about 10–6 Ma (Wang *et al.*, 2014). Taiwan emerged above sea level in the late Miocene (10–5 Ma; Huang *et al.*, 2006) during the same period, coincident with the formation of the Ryukyu Arc. The major components of the Taiwan-Ryukyu archipelago were uplifted in the late Miocene and arrived at their modern configuration in the early Pliocene (Sibuet *et al.*, 1995). This geological process provided ample opportunities for the previously isolated biota to spread southward to Taiwan. It also allowed sufficient time for allopatric speciation on these islands to generate island endemics. This hypothesis, which we term the Pre-Pleistocene Isolation (PPI) hypothesis, predicts that lineages from Ryukyu may have been the first to diverge, and that lineages from southern islands and Taiwan should be younger and more recently derived. Kizaki & Oshiro (1977) have suggested a similar hypothesis based on their reconstruction of the land mass connection; empirical evidence congruent with the PPI hypothesis (diversification before the Pleistocene) had been reported from terrestrial organisms such as reptiles (Honda *et al.*, 2014), amphibians (Matsui *et al.*, 2005), and insects (Maekawa *et al.*, 1999). However, explicit investigations of the PPI hypothesis were not emphasized in those earlier studies.

Spiders of the genus *Macrothele* are most diverse in East Asia and Central Africa; only one species occurs in Europe (Arnedo & Ferrández, 2007). A total of six recognized species occur on the Taiwan-Ryukyu Archipelago. The *Macrothele taiwanensis* species group includes *M. amamiensis* (Amami Island), *M. yaginumai* (Ishigaki and Iriomote) and *M. taiwanensis* (Taiwan) (Shimojana & Haupt, 1998). These ground-dwelling spiders have very limited dispersal capacities, and only adult males are migratory over short distances in association with reproductive effort. Morphologically, they are extremely conservative and are only identifiable by subtle differences in male genitalia (Shimojana & Haupt, 1998). Their multi-island distribution and limited migratory abilities make the *M. taiwanensis* species group an ideal study system

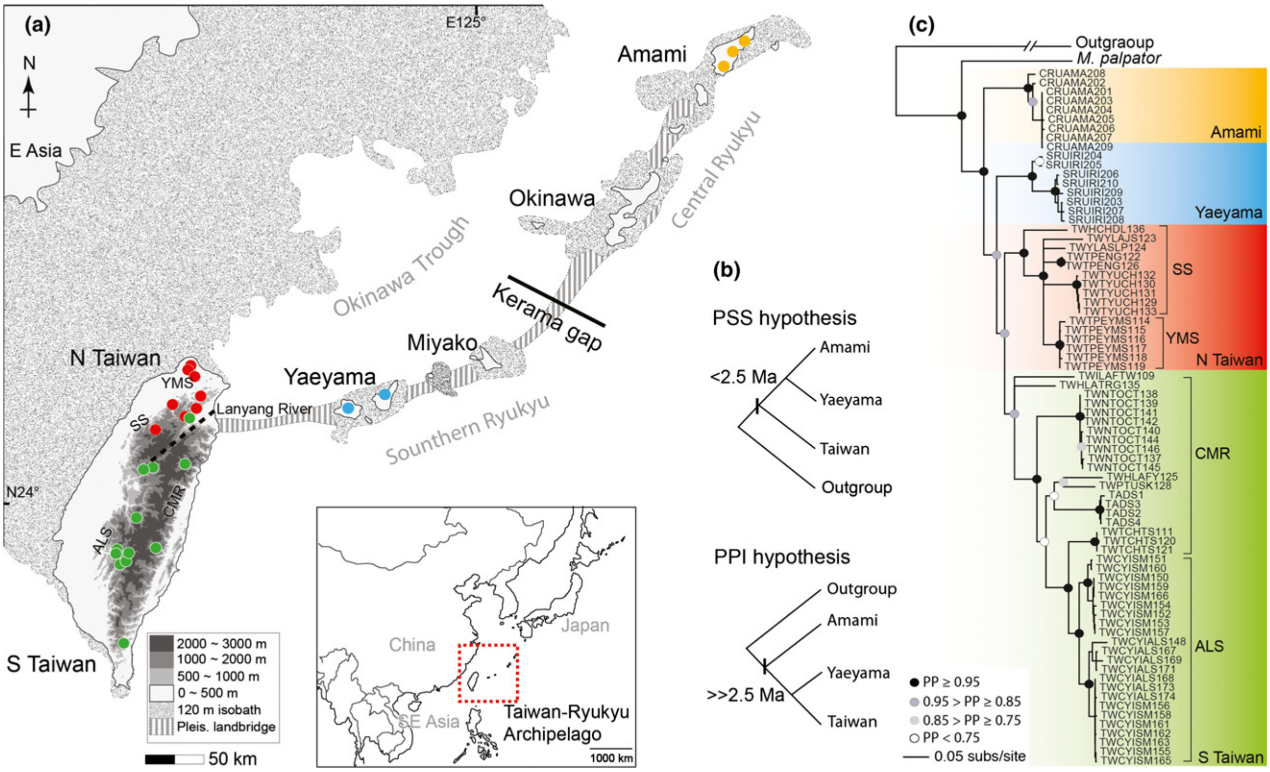

Figure 1 Collection sites, hypotheses, and the phylogeny of the *Macrothele taiwanensis* species group. (a) Map of the Taiwan-Ryukyu Archipelago. The 120-metre isobath contours, possible land bridges in the Pleistocene (Kimura, 2000), current island boundaries, and variable elevations of Taiwan are presented in incremental shades of gray. (b) The Pleistocene stepping stone (PSS) and Pre-Pleistocene isolation (PPS) hypotheses (experimental topologies, indicating the polarity of diversification); (c) Preferred topology from a MrBayes 50% majority consensus tree. Collection sites colour-coded to match tree (Fig. 1a).

with which to test the impacts of palaeo-geographical barriers on processes of evolutionary diversification in the Taiwan-Ryukyu Archipelago.

Here, we used Bayesian methods to test the predictions of the PSS and PPI hypotheses (Fig. 1b) as explanations for patterns of diversification in the *M. taiwanensis* species group. The PSS hypothesis predicts that diversification should have occurred within 2.5 Myr, via ancestral lineages derived from Taiwan, and that the Pleistocene climate fluctuations should have drastically affected population demography. In contrast, the PPI hypothesis predicts that the divergence times among island lineages should be significantly earlier than the Pleistocene, derived from ancestral Ryukyu lineages, and that past population demography should have been stable during the Pleistocene. We used two species delimitation statistics to determine whether potential cryptic species exist among morphologically similar but geographically isolated populations. Our results demonstrate support for the PPI hypothesis as an alternative explanation for shaping the biodiversity in the Taiwan-Ryukyu Archipelago. Although rarely discussed previously, we suspect that this alternative mechanism may be a more general and tractable explanation for the high level of faunal endemism in the Ryukyu faunal region, especially in organisms with relatively limited dispersal abilities.

MATERIALS AND METHODS

Taxon sampling

We sequenced 76 individuals of the *M. taiwanensis* species group. We collected *M. amamiensis* on Amami Island and *M. yaginumai* on Yaeyama Islands. The *M. taiwanensis* populations were collected in the northern mountains near Yanmingshang (YMS) and Shiueshang (SS), in the southern mountains near Alishang (ALS), and along the Central Mountain Range (CMR) (Fig. 1a). We chose *M. palpator* (a continental species closely related to the ingroup), *M. gigas* (a continental and Taiwan-Ryukyu species), *M. holsti* (an species endemic to lowland Taiwan) and *M. calpeiana* (a European species) as outgroup. We also followed Arnedo & Ferrández (2007) in using *Atrax robustus* (an Australian funnel web spider) as the outgroup for *Macrothele* (see Appendix S1 in Supporting Information). Data matrices were deposited in TreeBase (No.15694).

Molecular protocol and phylogeny estimation

We extracted genomic DNA using Qiagen kits (Valencia, CA, USA) following commercial protocols. We sampled 839 base pairs of mitochondrial cytochrome oxidase I subunit

(*COI*) and 457 base pairs of mitochondrial *16S* rRNA (*16S*) sequence following the polymerase chain reaction, sequencing, and alignment protocols in Su *et al.* (2011).

MrBayes 3.2.1 (Ronquist & Huelsenbeck, 2003) was used to reconstruct the Bayesian phylogenetic trees based on *COI*, *16S* and concatenated data sets. *COI* sequences were partitioned into three codon positions. We used the Akaike information criterion (AIC), as implemented in jModelTest 2.1.7 (Darriba *et al.*, 2012) to identify the best-fit substitution model for each partition (*COI* codon position1: TIM2+I+G; position2: TrN+G; position3: GTR+G; and *16S*: TIM3+I+G). The analyses each comprised two independent Markov chain Monte Carlo (MCMC) chains, with 1×10^8 generations per run, 1×10^3 generations/tree sampling frequency, and discarding the first 25% of the sampled trees as 'burn-in'. We visually inspected the likelihood scores of trees in Tracer 1.6 (Rambaut & Drummond, 2009). Posterior probabilities (PP) of clades were computed from the remaining trees to produce a consensus tree for each data set.

We performed maximum likelihood analyses for each data set in garli 2.0 (Zwickl, 2006) using the same partitioning strategy and specified the same models of sequence evolution as in our Bayesian analyses. We performed 1×10^3 bootstrap replications and summarized the bootstrap consensus tree using 50% majority rule to evaluate the support for each node.

Topology tests

We constructed null topologies under the PSS hypothesis predictions, namely that genealogical relationships of species should be (Taiwan, (Yaeyama, Amami)). Alternatively, the PPI hypothesis predicts a topological arrangement of (Amami, (Yaeyama, Taiwan)). We followed Siler *et al.* (2013) and used a Bayesian hypothesis-testing approach in which the pool of post 25% burn-in trees generated from MrBayes were filtered in paup* 4.0 (Swofford, 2002) with constrained topologies according to the aforementioned predictions. The proportion of posterior trees in each topology was then used to calculate the posterior probability of each topology. We rejected topologies with a probability ≤ 0.05.

Divergence time estimation

To evaluate timeframe of diversification in Taiwan-Ryukyu endemic *Macrothele* species, we used five different schemes (Table 1): (1) the 1.55 Ma timeframe, which corresponds to the opening of South Okinawa Trough, in Osozawa *et al.* (2012) of Taiwan-Ryukyu diversification, (2) a Mygalomorphae mitochondrial molecular clock (4.0% Myr^{-1}, Bond *et al.*, 2001), (3) the initial opening time of the Okinawa Trough (Wang *et al.*, 2014), (4) the arthropod mitochondrial molecular clock (2.3% Myr^{-1}; Brower, 1994) and (5) Opatova & Arnedo's (2014) estimated origin of the Asian *Macrothele*+*M. calpeiana* clade (40.77 Ma, 95% highest probability density, or 95% highest posterior density [HPD] = 58.0–26.6). Finally, we compared the fit of results to the geological record, and evaluated support for feasibility of each of these temporal frameworks.

All divergence times based on the five schemes were estimated using the concatenated data matrix under a gene-tree framework in beast 1.8.0 (Drummond *et al.*, 2012). We unlinked the substitution rates and the clock models of each gene, set up the appropriate substitution model, and used a variety of priors (Table 1) for each time calibration scheme. We combined two independent runs of a scheme in beast with 50% burn-in for each run. Each MCMC chain length was 6×10^8 with a sampling frequency of 1×10^3, which provided sufficient effective sample sizes (ESS > 200). Results of independent runs were input into Tracer 1.6 to diagnose convergence. A final maximum clade credibility tree was generated using TreeAnnotator 1.8.0.

Ancestral range estimation

Ancestral ranges were reconstructed using a Markov discrete phylogeographical model with Bayesian Stochastic Search Variable Selection, BSSVS (Lemey *et al.*, 2009), implemented in beast. This method simultaneously assesses the uncertainty associated with phylogenies and ancestral ranges. We utilized the same priors, chain length and burn-in used in divergence time estimation with the addition of coded geographical ranges for each of the lineages. Four distributional ranges—Amami, Yaeyama, northern Taiwan (SS+YMS) and southern Taiwan (CMR+ALS)—were encoded for each terminal, based on barriers formed by mountain ranges and island boundaries. Outgroup areas were set as their current ranges.

We also used the r package, BioGeoBEARS (Matzke, 2013a), to estimate ancestral ranges and infer speciation modes. This method implements three likelihood-based models: Dispersal-Extinction-Cladogenesis (DEC; Ree & Smith, 2008), the likelihood version of dispersal–vicariance (DIVA; Ronquist, 1997; herein DIVALIKE), and the likelihood version of BayArea model (Landis *et al.*, 2013; herein BAYAREALIKE). In each model, an additional *j* parameter for founder events was added; thus, a total of six models resulted (Matzke, 2013b). We used the final ultrametric tree generated from beast analyses with the same range coding as BSSVS analysis but kept *M. palpator* (the closest relative of our focal clade) as the outgroup. We compared the fit of each model using the AIC weighted approach (Burnham & Anderson, 2002). Ancestral ranges and the biogeographical events were then estimated under the best-fit model.

Species delimitation

We evaluated species boundaries using two Bayesian species delimitation methods. In the Bayesian implementation of the

Table 1 Descriptions of time calibrations employed in this study.

Scheme	Calibration	Input prior in BEAST	Probability distribution	Calibrated node	Asian+*M. calpeiana*		Taiwan-Ryukyu		Estimated *COI* mean rate ($site^{-1}$ Myr^{-1})	Estimated *16S* mean rate ($site^{-1}$ Myr^{-1})
					Age (Ma)	95% HPD	Age (Ma)	95% HPD		
Opening of South Okinawa Through (Osozawa *et al.*, 2012)	1.55 Ma	Mean = 1.55 With arbitrary SD = ± 1.0	Normal	Taiwan-Ryukyu group	2.8	3.50–2.08	1.5	2.16–1.45	0.154	0.0934
Mygalomorphae mitochondrial clock (Bond *et al.*, 2001)	4.00% Myr^{-1}	0.0200 $site^{-1}$ Myr^{-1}	Lognormal	Whole tree	15.4	19.79–10.95	9.3	11.59–6.99	NA†	NA†
Initial opening of Okinawa Through (Wang *et al.*, 2014)	6.00–10.00 Ma	Mean = 8.00 SD = ± 1.20 (puts 10–6 Ma in 95% HPD)	Normal	Taiwan-Ryukyu group	14.4	19.83–9.20	7.8	10.15–5.50	0.0299	0.0181
Arthropod mitochondrial clock (Brower, 1994)	2.30% Myr^{-1}	0.0115 $site^{-1}$ Myr^{-1}	Lognormal	Whole tree	26.6	34.47–18.67	16.1	20.18–12.18	NA†	NA†
Origin of Asian+*M. calpeiana* clade(Opatova & Arnedo, 2014)	40.77 Ma*	Mean = 40.77 SD = ± 8.00 (puts 58–26 Ma in 95% HPD)	Normal	Asian+*M. calpeiana*	36.9	49.00–26.60	19.2	27.86–12.14	0.0124	0.0075

*Opatova & Arnedo (2014) used a Jurassic fossil to calibrate Mygalomorphae phylogeny and estimated that Asian+*M. calpeiana* clade originated in 40.77 Ma (95% highest posterior density [HPD] = 58.00–26.66 Ma).

†NA: we did not estimate the substitution rate if a molecular clock prior was used.

General Mixed Yule Coalescent (bGMYC, Reid & Carstens, 2012) analyses, we used collection sites as the upper bound of the putative number of species. However, if the distances between two sites were less than 50 km, we combined them. In Taiwan, based on the geographical proximities of collection sites, we proposed the YMS sites as one species, and the SS sites as two species, the ALS sites as a species and the scattered sites along the CMR as six species. Together with *M. yaginumai* and *M. amamiensis*, and five outgroups, we proposed a total of 17 species in bGMYC analyses.

The bGMYC package in R implements a Bayesian version of the GMYC model (Pons *et al.*, 2006) to account for uncertainty of tree topologies in species delimitation. We obtained a subsample of 1×10^2 trees from the post-burn-in trees generated in BEAST analyses. Under each tree topology, 5×10^4 MCMC generations were run with a sampling frequency of 1×10^2 and 80% burn-in. The resulting 1×10^4 samples were used to calculate the marginal probabilities of species identities. Instead of the default prior settings, we used 17 species as the upper bound and raised the Yule and coalescent rate change parameters (scale vector, 20, 4, 2) to keep the acceptance rates of each parameter ranging from 0.2 to 0.8 as suggested by Reid & Carstens (2012).

In Bayesian Phylogenetics and Phylogeography (BPP, Yang & Rannala, 2010) analyses, we employed current island boundaries and distinct mountain ranges within Taiwan as our guide to form our hypothesis of species boundaries. BPP uses a reversible-jump MCMC (rjMCMC) algorithm to generate a posterior probability for each proposed speciation event on the user-specified guide trees, and to accommodate lineage sorting of ancestral polymorphism. Two different combinations of the gamma priors for population size parameters (θs) and gamma priors to age of the root in the species tree (τ_0s) were employed to estimate the posterior probabilities of the models (Yang & Rannala, 2010): (1) $\theta \sim G$ (1, 10) and $\tau_0 \sim G$ (2, 2,000), representing small population sizes and deep divergence for models with more species, and (2) $\theta \sim G$ (2, 2,000) and $\tau_0 \sim G$ (1, 10), representing large population sizes and shallow divergence for models with fewer species. We used a variety of fine-tuning parameters and multiple runs were performed to confirm consistency between runs. Each run was performed for 1×10^5 generations, with a sampling frequency of five and a burn-in of 2×10^4 generations.

Population demographic changes

We used Extended Bayesian Skyline Plots (EBSP; Heled & Drummond, 2008) to detect historical population demographic changes. This method uses multi-locus data and makes Bayesian coalescent inferences of each locus to reconstruct the population demographic history. We conducted EBSP on the Amami, Yaeyama, northern Taiwan, and southern Taiwan lineages using two mitochondrial gene regions with sample sizes ranging from eight to 32. We also assessed recent population size expansion by calculating mismatch distributions in each gene region of the four lineages using ARLEQUIN 3.5 (Excoffier & Lischer, 2010).

RESULTS

Phylogenetic analyses

Bayesian analyses (Fig. 1c and see Appendix S2) of our concatenated dataset demonstrated that *M. amamiensis* from Amami Island is monophyletic (PP: MRBAYES = 1.00, BEAST = 1.00) and diverged first. This was followed by the monophyletic *M. yaginumai* from Yaeyama Islands (PP: MRBAYES = 1.00, BEAST = 1.00), which is moderately supported as sister to *M. taiwanensis* populations from Taiwan (PP: MRBAYES = 0.89, BEAST = 0.84). The latter consists of two large populations divided by the Lanyang River. The monophyletic northern Taiwan population consisted of individuals from YMS and SS (PP: MRBAYES = 1.00, BEAST = 1.00), and the southern Taiwan clade included individuals from CMR and ALS and an individual from SS near Lanyang River (PP: MRBAYES = 0.94, BEAST = 0.97). Within the northern Taiwan population, the individuals from YMS formed a monophyletic clade (PP: MRBAYES = 0.99, BEAST = 0.94) while individuals from SS lacked monophyletic sub-grouping. In southern Taiwan, the small populations scattered along CMR did not form a clade but the individuals in ALS were monophyletic (PP: MRBAYES = 1.00, BEAST = 1.00). Similar phylogeographical patterns were also supported by likelihood analyses and the analyses of individual genes but they did not resolve relationships among the four large populations (see Appendix S2).

Divergence time estimation

When the Mygalomorphae *COI* molecular clock (4% Myr^{-1}) was applied, we reconstructed the oldest ingroup node as 9.3 Ma (95% HPD = 11.59–6.99), which approximates the estimated time (7.8 Ma, 95% HPD = 10.15–5.50) inferred when using the prior of the initial opening of the Okinawa Trough (10–6 Ma, Wang *et al.*, 2014). The results using the priors of the arthropod mitochondrial clock (16.1 Ma, 95% HPD = 20.18–12.18) and the origin of the Asian+*M. calpeiana* clade (Opatova & Arnedo, 2014; 19.2 Ma, 95% HPD = 27.86–12.14) produced a mid-Miocene age estimate for the Taiwan-Ryukyu clade. In all cases, endemic island lineages were dated before the Pleistocene except when using 1.55 Ma (Osozawa *et al.*, 2012) as a prior (Table 1 and Fig. 2). Using the 1.55 Ma prior for calibration also resulted in excessively high substitution rates in both *COI* (7.7 × faster than 4% Myr^{-1}; 13.4 × faster than 2.3% Myr^{-1}) and *16S* (4.7 × faster than faster than 4% Myr^{-1}; 8.1 × faster than 2.3% Myr^{-1}). Using the 1.55 Ma prior also resulted in the origin of the Asian+*M. calpeiana* clade to be estimated at 2.8 Ma (95% HPD = 3.50–2.80), which is *c.* 38 Myr younger than the preferred estimate from Opatova & Arnedo (2014) (Table 1).

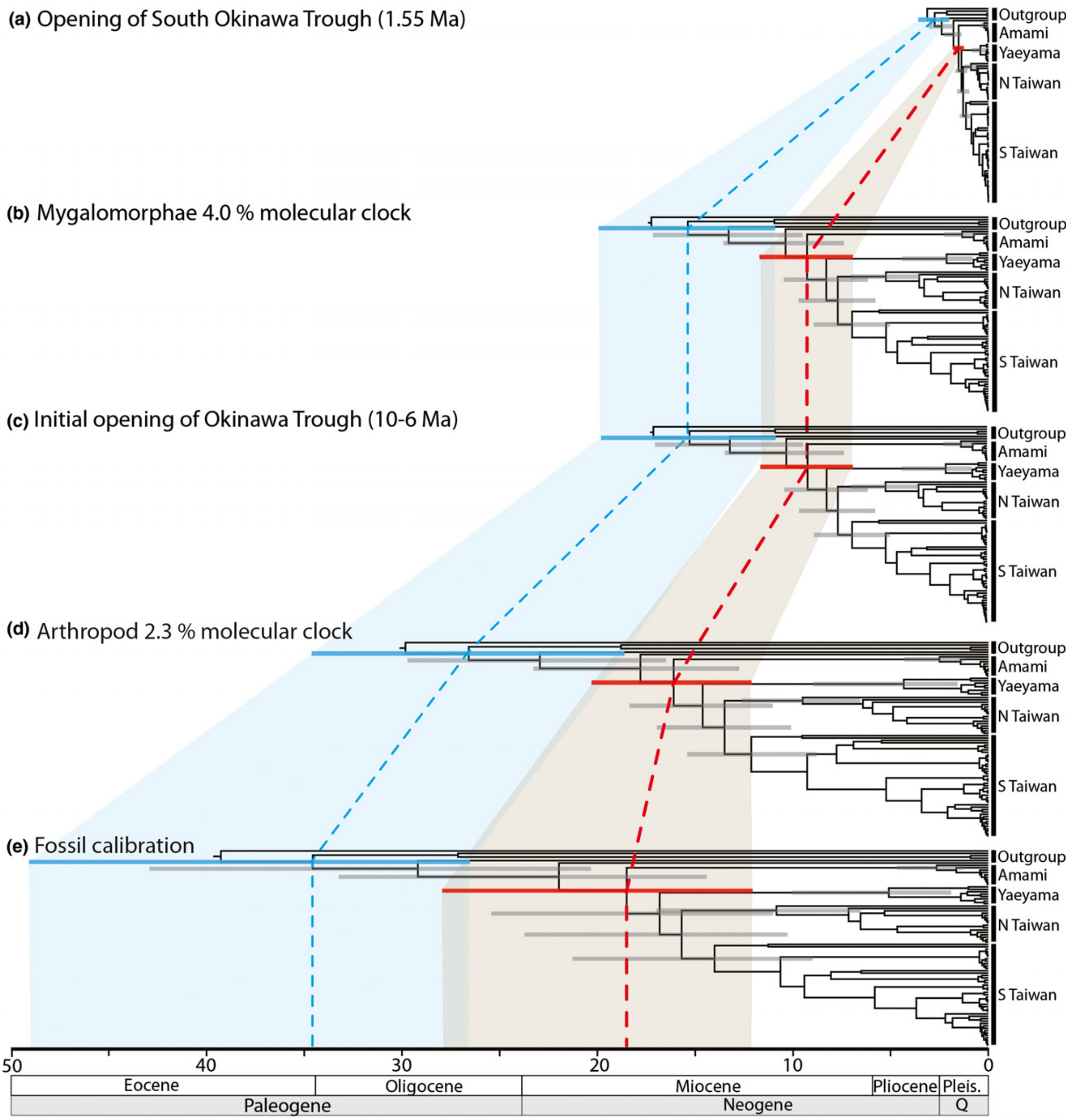

Figure 2 Divergence time estimation based on five calibration schemes (Table 1). (a) Opening of the South Okinawa Trough (1.55 Ma) suggested in Osozawa *et al.* (2012); (b) The Mygalomorphae (4.0% Myr^{-1}) molecular clock; (c) Initial opening of the Okinawa Trough (10–6 Ma); (d) The 2.3% Myr^{-1} arthropod molecular clock; (e) Secondary calibration utilizing the Asian+*M. calpeiana* clade (40.77 Ma).

Topology tests

Our test failed to reject (PP = 0.763) the constrained topology based on the PPI hypothesis [Amami, (Yaeyama, Taiwan)]. On the contrary, the data rejected the constrained topology [Taiwan, (Yaeyama, Amami)] of the PSS hypothesis (PP = 0.002). The results of our Bayesian tests of alternative topologies support a diversification sequence starting first from northern Amami, then Yaeyama, and finally Taiwanese lineages (Fig. 3a).

Ancestral range estimation

The BSSVS ancestral range showed the highest posterior probability for northern Taiwan as the ancestral range for *M. taiwanensis* (Fig. 3b). The ancestral range for the lineage

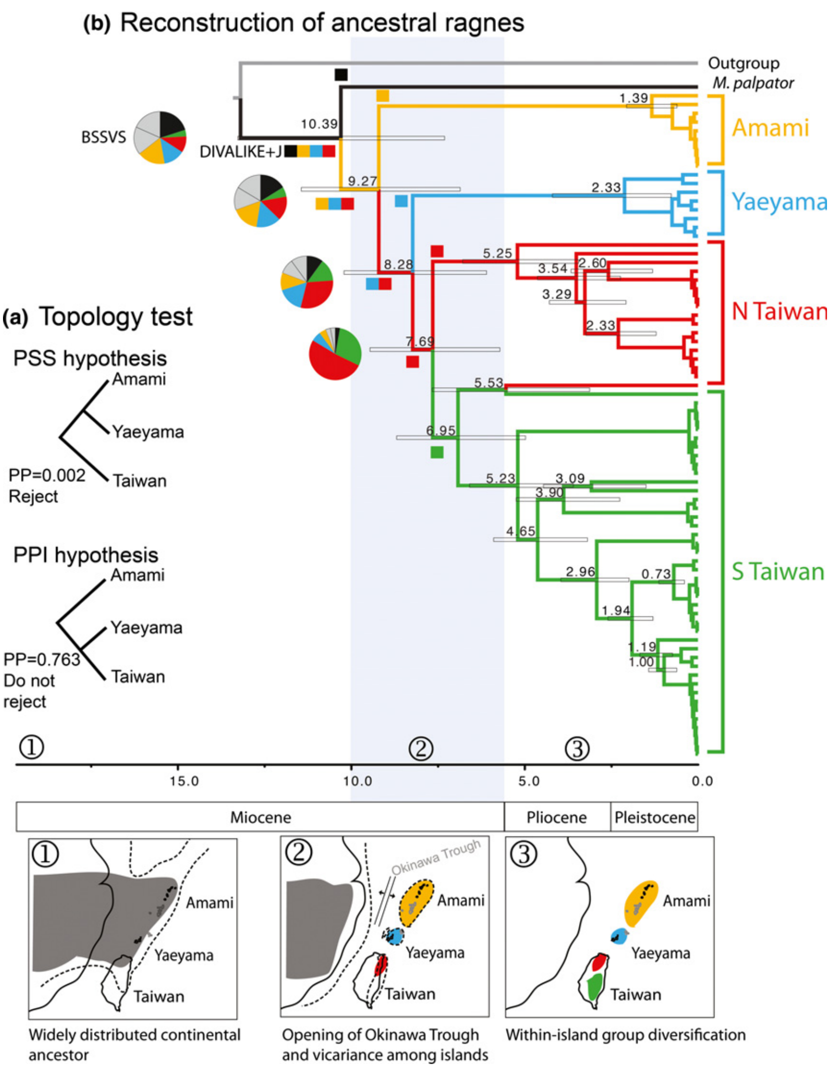

Figure 3 Topology tests and ancestral range estimation. (a) A Bayesian topology test rejected the Pleistocene Stepping Stone topology but not the Pre-Pleistocene Isolation hypothesis. (b) Bayesian reconstructions of ancestral ranges using Bayesian Stochastic Search Variable Selection and BioGeoBEARS. Branch colour indicates the reconstructed ancestral range of highest posterior probability. Pie charts depict the relative probability of ancestral range in BSSVS; boxes show ancestral areas using BioGeoBEARS. Along time-scale, circle 1 to 3 denote possible processes of diversification. Geological reconstructions based on Kizaki & Oshiro (1977) and Wang *et al.* (2014).

leading to Taiwan and Yaeyama Islands was inferred to be northern Taiwan. At the deepest node, the estimated ancestral range for the clade was inferred to be Amami Island (Fig. 3b). These results agree with the Bayesian topology test, indicating a north-to-south diversification sequence.

The BioGeoBEARS analyses showed the best-fit biogeographical model was DIVALIKE+*J* (Table 2). The ancestral range estimate based on this model inferred a process of vicariance for diversification among current landmasses. The estimated ancestral ranges at the node connecting to the outgroup indicated the Eurasian Continent, Amami, Yaeyama, and Taiwan at *c.* 10.4 Ma if a 4% clock prior was applied. Following the inference of the first vicariance event (*c.* 9.3 Ma), the Amami Island population was isolated. The

Table 2 Results of BioGeoBEARS estimation of ancestral ranges, using a model-selection approach to identify the appropriate biogeographical model for inference of range evolution in species of the Taiwan-Ryukyu endemic *M. taiwanensis* group. Model parameters include dispersal (*d*) and extinction (*e*) for likelihood version of dispersal–vicariance (DIVALIKE), Dispersal–Extinction–Cladogenesis (DEC) and likelihood version of Area (BAYAREALIKE) models each with a founder parameter *j*, thus resulted six models for comparison using the Akaike information criterion (AIC) weight method (Burnham & Anderson, 2002).

Model	Log likelihood	Number of parameters	*d*	*e*	*j*	AIC	delta-AIC	AIC weight
DIVALIKE+*j*	−13.1643	3	1.00×10^{-12}	1.00×10^{-12}	0.02589718	32.3286	1.0000	0.76
DEC+*j*	−14.9387	3	1.00×10^{-12}	1.00×10^{-12}	0.02831284	35.8775	0.1696	0.13
DEC	−16.7622	2	2.64×10^{-3}	1.00×10^{-12}	0	37.5244	0.0744	0.06
DIVALIKE	−16.8959	2	5.34×10^{-3}	1.00×10^{-12}	0	37.7918	0.0651	0.05
BAYAREALIKE+*j*	−17.4834	3	1.00×10^{-7}	1.00×10^{-7}	0.04995876	40.9669	0.0133	0.01
BAYAREALIKE	−25.1266	2	5.36×10^{-3}	1.02×10^{-1}	0	54.2533	0.0000	0.00

Yaeyama Island population became isolated at a second inference of vicariance (*c.* 8.3 Ma). Finally, our analyses inferred a dispersal or founder event from northern Taiwan to southern Taiwan (*c.* 7.7 Ma) (Fig. 3b).

Species delimitation

Results of our BPP analyses agreed with the morphological determination that *M. amamiensis* on Amami (PP = 1.00) and *M. yaginumai* on Yaeyama islands (PP = 1.00) are valid species. For the lineages in Taiwan, BPP analyses additionally supported the recognition of individuals from northern Taiwan as one species (PP = 1.00). In southern Taiwan, the populations of ALS appear to form a species, as do individuals from CMR (PP = 1.00). However, the CMR population did not form a monophyletic lineage (Fig. 4). The result of at least five species was supported by bGMYC analyses, which also postulated the existence of many more species. In northern Taiwan, bGMYC identified two putative species, but the distributions of these putative species are not associated with separate mountain ranges. Samples from southern Taiwan were divided into multiple species, indicating that nearly every small population scattered along CMR could be considered as a distinct species (Fig. 4). The larger population in ALS (denoted with a green circle 6 in Fig. 4) with one collection site in CMR (green circle 3 in Fig. 4) formed another species. However, sampling was limited for most of the groups delimited along CMR (1–4 individuals per lineage).

Population demographic changes

The EBSP results suggested stable population sizes in Amami, Yaeyama, and the northern Taiwan lineages. The southern Taiwan lineages showed a population decline within the last million years (see Appendix S3). Mismatch distributions showed no apparent signal of recent population demographic changes estimated from either gene and were multi-modal (indicating stable, structured populations) for all the lineages.

DISCUSSION

Our results for sedentary funnel web spiders have identified a highly probable but rarely suggested process of faunal diversification in the Taiwan-Ryukyu Archipelago. This mechanism and temporal framework may aid in understanding diversification in other organisms with similarly limited dispersal abilities. However, we emphasize that the alternative Miocene hypothesis and the commonly invoked Pleistocene

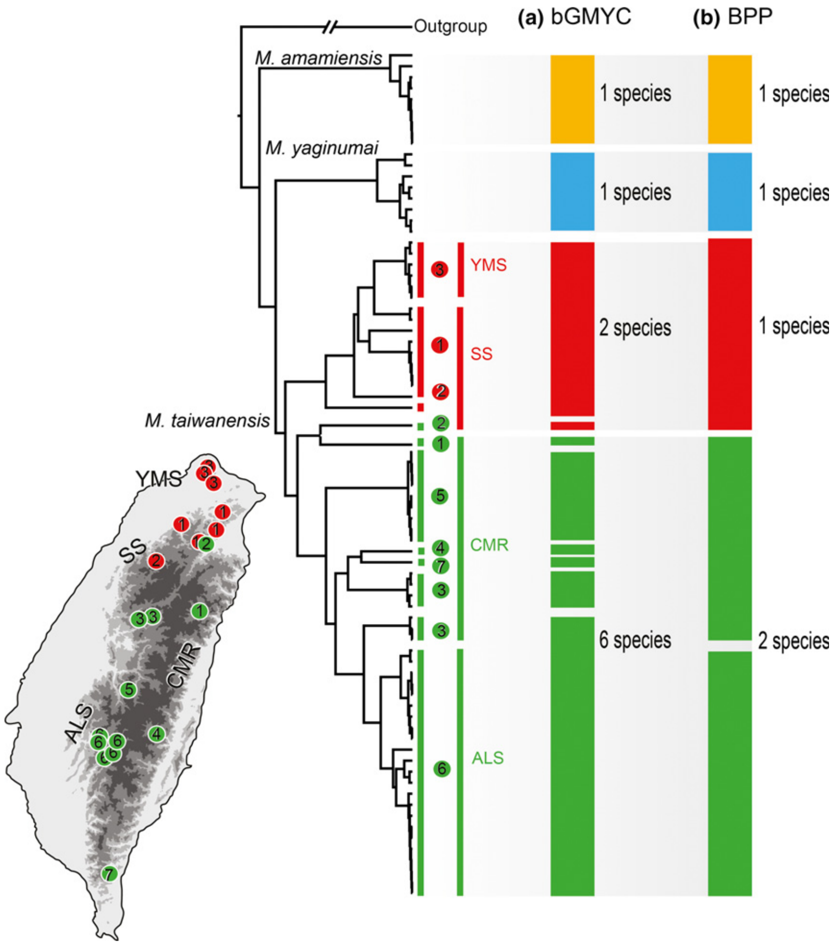

Figure 4 Bayesian species delimitation. Colour-coded circles indicate collection sites (Fig. 1). Numbers in circles indicate potential species, with names on nodes indicating current taxonomic treatment. Coloured blocks denote species membership (with a Posterior probabilities > 0.95). Vertical lines indicate potential species assignments and associated mountain ranges. (a) bGMYC species delimitation resulted in 10 putative species. (b) Bayesian Phylogenetics and Phylogeography species delimitation statistically detected five species (see text).

hypothesis are not mutually exclusive. Even in archipelagos with few islands and simple geological histories, phylogenies of co-distributed taxa are often topologically and/or temporally incongruent (Lomolino *et al.*, 2006), as might be expected from the diversity of life histories, generation times, and dispersal abilities (Oaks *et al.*, 2013). Results of our model-based phylogeographical analyses allow us to reject the hypothesis that the distributions of *M. taiwanensis* species are the result of Pleistocene geological events. Rather, in this case, it appears that the Miocene–Pliocene island isolation has disproportionately contributed to the accumulation of endemic biodiversity of these Taiwan-Ryukyu funnel web spiders.

The PSS hypothesis, frequently invoked for other terrestrial organisms (e.g. Chiang *et al.*, 2006; Nakamura *et al.*, 2010; Mitsui & Setoguchi, 2012; Muraji *et al.*, 2012), postulates that after ancestral lineages arrived in Taiwan (the first stepping stone) during the Pleistocene, and that genetic structure of populations may be shaped by subsequent northward dispersal events and stepwise isolation – possibly resulting in *in situ* speciation. However, our topological tests and diversification time estimates strongly rejected both Pleistocene diversification (Fig. 2) and a south-to-north progression (Fig. 3) for funnel web spiders.

Osozawa *et al.* (2012) reconstructed the palaeogeography of the Taiwan-Ryukyu Archipelago and suggested that central Ryukyu (Amami+Okinawa), southern Ryukyu (Yaeyama), and Taiwan were separated from the continent simultaneously within a 1.55 Ma timeframe; these authors thus invoked a vicariance explanation of biogeographical diversification in the archipelago. Our ancestral range estimation procedure is in accordance with the hypothesis of vicariance (and an associated interpretation of allopatric speciation). However, our molecular clock-based divergence time estimates (Table 1) and the estimates derived from the higher level phylogeny of *Macrothele* (Opatova & Arnedo, 2014) clearly are congruent with the Miocene diversification hypothesis. Additionally, when a Mygalomorphae 4.0% mitochondrial clock was applied, divergence time estimates agreed with results from analyses employing calibrations based on the initial opening of the Okinawa Trough. However, none clearly agreed with a simultaneous Pleistocene vicariance hypothesis (Osozawa *et al.*, 2012) (Fig. 2). Moreover, when the 1.55 Ma scenario was applied to the deepest node of our focal clade, the results required excessively and unrealistically high substitution rates in *COI* and *16S*. Additionally, under this assumption, the origin of the Asian+*M. calpeiana* clade was dated extremely early in the late Pliocene (Table 1). Clearly, results from funnel web spiders demonstrate further the incompatibility our data with the reconstruction suggested by Osozawa *et al.* (2012).

One additional point warrants discussion. Because the timing of the opening of the Okinawa Trough is controversial (Wang *et al.*, 2014) and geological reconstruction before the Pleistocene is both complex and patchy with respect to data type and informativeness (Osozawa *et al.*, 2012; Wang *et al.*, 2014), a robust geological reconstruction of the precise geological events during the Miocene–Pliocene is a priority for future geological studies. However, the absence of precise geological reconstructions does not negate the inference of diversification within this period, especially for taxa with sedentary life histories, which would be suggestive of diversification by palaeotransport (e.g. Opatova & Arnedo, 2014).

Our analyses of demographic change take a different approach to assess data for effects of Pleistocene events. Our results showed that relatively recent events in the Pleistocene did not impart an obvious demographic signature in the major lineages of the *M. taiwanensis* species group. In historical demographic analyses, older lineages of the northern parts of the archipelago are not characterized by recent population demographic changes through time. However, the younger southern Taiwan lineage, which demonstrated a recent population expansion, agrees with the BioGeoBEARS finding of dispersal and founder event speciation from northern to southern Taiwan.

Our species delimitation procedures with molecular data agree with the taxonomic inference based on morphology. Despite it being conceivable that species numbers in Taiwan could be over-estimated due to fast evolutionary rates of mitochondrial markers, our analyses identified at least three potential cryptic species. Because *M. taiwanensis* is now only distributed from 500 to 1,500 m a.s.l., we tested species boundaries within Taiwan employing distinct mountain ranges as our guide. The BPP and bGMYC results identified at least one putative species in northern Taiwan and two in southern Taiwan. Our divergence time estimates indicated a late Miocene to Pliocene timeframe (ca. 7–5 Myr) for the first speciation event within Taiwan, corresponding to the uplift of the northern portions of this island (Fig. 3). Subsequent diversification may have then occurred along CMR and ALS after 5 Ma, suggesting that speciation within Taiwan may have resulted from continuous orogenesis during the late Miocene to the Pliocene (Huang *et al.*, 2006) as a consequence of isolation in higher elevation montane habitats.

Our results from funnel web spiders argue for a relatively ancient history of diversification, consistent with a Miocene–Pliocene island isolation sequence, as one of the probable mechanisms for high levels of endemism in the Taiwan-Ryukyu Archipelago fauna. However, despite support from a few biogeographical studies (Maekawa *et al.*, 1999; Honda *et al.*, 2014), this alternative explanation largely has been ignored in recent studies that have instead focused on Pleistocene diversification scenarios. The results of our study draw attention to the need for continued future study of organisms with high habitat fidelity to test predictions of pre-Pleistocene hypotheses, corresponding to the geological timeframe for the initial opening of the Okinawa Trough. We predict once sufficient numbers of endemic taxa from the Taiwan-Ryukyu Archipelago are thoroughly considered, timing of diversification estimates will empirically cluster into three categories that correspond to the three stages of the extension of the Okinawa Trough (Sibuet *et al.*, 1995; Wang *et al.*, 2014). Clearly these are fertile grounds for future research in comparative, multi-taxon biogeography (Chiang & Schaal, 2006; Brown *et al.*, 2013; Oaks *et al.*, 2013).

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Appendix S1 Sample list.
Appendix S2 Detailed Bayesian and likelihood trees.
Appendix S3 Demographic changes.

BIOSKETCH

Yong-Chao Su is interested in the biogeography of Southeast Asia.

Author contributions: I.-M.T., Y.-C.S., and Y.-H.C. designed the study and conducted field work. Y.-C.S. generated molecular data and conducted analyses with input from R.M.B. and C.-P.L.; Y.-C.S. and R.M.B. led the writing.

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