ORIGINAL ARTICLE

Pre-Pleistocene geological events shaping diversification and distribution of primitively segmented spiders on East Asian margins

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ABSTRACT

Aim Phylogeographical research in the East Asian continent and islands (East Asian Margins, or EAM) suggests predominant Pleistocene over-water dispersal events from continent to islands, but more ancient biogeographical patterns in EAM remain obscure. We explored biogeographical histories and population genetic structures of the primitively segmented spiders, *Heptathela* and *Ryuthela* (Liphistiidae), broadly codistributed across EAM islands. To test competing biogeographical hypotheses, we looked for geohistoric events that may have shaped their distributional patterns.

Location Kyushu and Ryukyus.

Methods We sampled 17 *Heptathela* and *Ryuthela* species on 10 out of 12 EAM islands and sequenced fragments of two mitochondrial and three nuclear genes. We performed phylogenetic, network, time-calibrated and biogeographical analyses to identify lineages, estimate their divergence times, and reconstruct ancestral ranges. We also assessed genetic diversity and historic demography of each lineage.

Results Phylogenetic origin of *Heptathela* + *Ryuthela* is estimated at 26.6–18.4 Ma (95% HPD) when EAM islands became separated from the continent. The crown ages of *Heptathela* (18.0–10.7 Ma) and *Ryuthela* (17.8–8.7 Ma) coincide with the formation of Japan Sea and Okinawa Trough respectively. A split within *Ryuthela* (13.9–6.0 Ma) coincides with the opening of the Kerama Gap, but a split within *Heptathela* (18.0–10.7 Ma) pre-dates the formation of the Tokara Gap. Speciation within *Heptathela* and *Ryuthela* is relatively recent (6.0–0.3 Ma). Population genetic and demographic results suggest interrupted gene flow within and among islands, with most species exhibiting stable past populations.

Main conclusions Historic biogeographical reconstructions strongly suggest predominant vicariant origins of EAM island liphistiids. Restricted gene flow following barrier formation is the dominant speciation force in these spiders. As they never seem to cross bodies of water, their past range expansion, successive fragmentation of EAM, and other vicariant events, must have shaped the detected genetic boundaries among and within islands.

Keywords

biogeography, Cenozoic, continental islands, dispersal, Kyushu, phylogeography, Ryukyus, vicariance

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INTRODUCTION

Islands represent natural laboratories for studying biogeography and evolution because of their complex geological history, well-defined boundaries, limited gene flow and high endemicity (Emerson, 2002; Whittaker & Fernández-Palacios, 2007; Losos & Ricklefs, 2009; Clouse & Giribet, 2010; Lomolino *et al.*, 2010). In the Cenozoic, the Ryukyu and Japanese archipelagos, the island arcs of the East Asian margins (EAM) underwent extensive changes in land configuration associated with block accretions and rotations as well as volcanism through interactions of the Eurasian, Indian, Pacific, and Philippine Sea tectonic plates (Otofuji *et al.*, 1985, 1991; Kano *et al.*, 1991; Ren *et al.*, 2002; Itoh *et al.*, 2006). The most significant tectonic events through the Eocene and early Pliocene were the establishment of the main islands of the Japanese Archipelago, the Ryukyu Archipelago, and the opening of a series of linked marginal seas (Japan Sea, Yellow Sea and East China Sea). Most Japanese and Ryukyu islands are thus fragmented continental islands. As one of most threatened biodiversity hotspots in the world (Myers *et al.*, 2000; Crowe *et al.*, 2006), EAM islands harbour diverse and largely endemic floras and faunas.

Several competing geological hypotheses explain the separation of the Japanese and Ryukyu archipelagos from mainland Asia. Kyushu was separated from the continent during the Miocene opening of the Japan Sea (23–15 Ma; Kizaki & Oshiro, 1977, 1980; Otofuji *et al.*, 1985, 1991). According to one hypothesis, the Ryukyu archipelago, which lies between Kyushu and Taiwan and forms the continuous Ryukyu Cordillera (Fig. 1), became gradually isolated from the Asian continent in the late Miocene (Ota, 1998; Kimura, 2000, 2003; Otsuka & Takahashi, 2000), but the exact timing of the opening of the Okinawa Trough ranges 10–6 Ma (Kimura, 2000, 2003; Sibuet & Hsu, 2004). Another hypothesis postulates the break of the southern Ryukyu islands from the continent in the Miocene (23–5.3 Ma), while the remaining Ryukyus were still part of Asia (Ujiié *et al.*, 1991; Ujiié & Nakamura, 1996). Despite the widely estimated timing from late Miocene to late Pliocene (~10 to 1.5 Ma), studies agree on the creation of two deep-water passages through the island arc, the Tokara Gap and the Kerama Gap (Fig. 1; Hikida & Ota, 1997; Ota, 1998). As a consequence of these events, the Ryukyu archipelago is biotically and geologically divided into three island groups (Ota, 1998; Kimura, 2000, 2003; Fig. 1): (1) the northern Ryukyus, composed of the Osumi Group and Tokara islands that faunistically resemble the main Japanese islands; (2) the central Ryukyus, consisting of the southern Tokara island, the Amami- and the Okinawa Group, harbouring highly endemic biotas; and (3) the southern Ryukyus, including the Miyako- and the Yaeyama Group. Unlike the central Ryukyus that have remained isolated since the initial openings of the two gaps between the late Miocene and late Pliocene (Hikida & Ota, 1997; Ota, 1998; Takahashi *et al.*, 2008), the northern and southern Ryukyus were repeatedly connected during Neogene to Kyushu and south-eastern China via Taiwan respectively (Ota, 1998). Some studies, however, report a shared biogeographical history of the central and southern Ryukyus (Takahashi *et al.*, 2008; Adler *et al.*, 2013).

Reconstruction of biogeographical histories of dispersal-limited organisms can be used to test such complex geological hypotheses. Phylogeographical research in EAM islands that involved a wide range of plants (e.g., Chiang & Schaal, 2006; Fan *et al.*, 2013) and animals (e.g., Igawa *et al.*, 2006; Aoki *et al.*, 2008; Li *et al.*, 2011; Adler *et al.*, 2013) focused on relatively young taxa of narrowly defined regions, or explored few populations. The recovered dominant biogeographical process from these studies is Pleistocene over-water dispersal from the continent to EAM islands. Such dispersal-dominated biogeographical histories offer limited tests of geological hypotheses and the evolutionary origins and geographical patterns of biota in EAM islands before the Pleistocene remain largely unexplored. To date, studies of vicariance/dispersal in the EAM islands have yielded ambiguous support for geological hypotheses, often interlaced with recent dispersals and extinctions that can disguise older patterns (Sanmartín & Ronquist, 2004; Upchurch, 2008; Sklenarova *et al.*, 2013).

Arachnids with ancient origins, limited dispersal abilities, and restricted habitats are good models for testing historical biogeographical hypotheses (Boyer *et al.*, 2007; Clouse & Giribet, 2010; Hedin *et al.*, 2013; Opatova & Arnedo, 2014a, b), and these include the primitively segmented spiders, family Liphistiidae (Xu *et al.*, 2015a). Liphistiids belong to the suborder Mesothelae dating back at least to the late Carboniferous with an Euramerian origin (295 Ma, Bristowe, 1976; Platnick & Gertsch, 1976; Selden, 1996; Haupt, 2003). This group represents 'living fossils' due to their retention of plesiomorphic traits such as vestigial abdominal segmentation, lost in all other spiders. Being relatively large, ground-dwelling spiders that build long-lasting trapdoor burrows and typically disperse only very short distances, they are not known to colonize land over water (Xu *et al.*, 2015a). Our prior study established that since the ancient origin of Mesothelae, the lineage must have undertaken a long, eastward, over-land trek with numerous extinctions to have reached the Asian origin of the family Liphistiidae in the Palaeogene (Xu *et al.*, 2015a). In contrast, the extant liphistiid genera diversified relatively recently, in the Cenozoic (Xu *et al.*, 2015a). Liphistiidae contains 91 species in eight genera confined to East and South-east Asia (World Spider Catalog, 2015; Xu *et al.*, 2015b). Of these, *Heptathela* Kishida, 1923 and *Ryuthela* Haupt, 1983 exclusively inhabit the EAM islands with 15 species. Liphistiid natural history, diversity and distributions make them excellent models for testing historical biogeographical hypotheses, and we here explore historical biogeography of these severely dispersal-limited spiders by shifting our focus from the genus level (Xu *et al.*, 2015a) to the species and population levels using the species restricted to EAM islands. *Heptathela* and *Ryuthela* have endemic species each confined to one (very rarely two) of
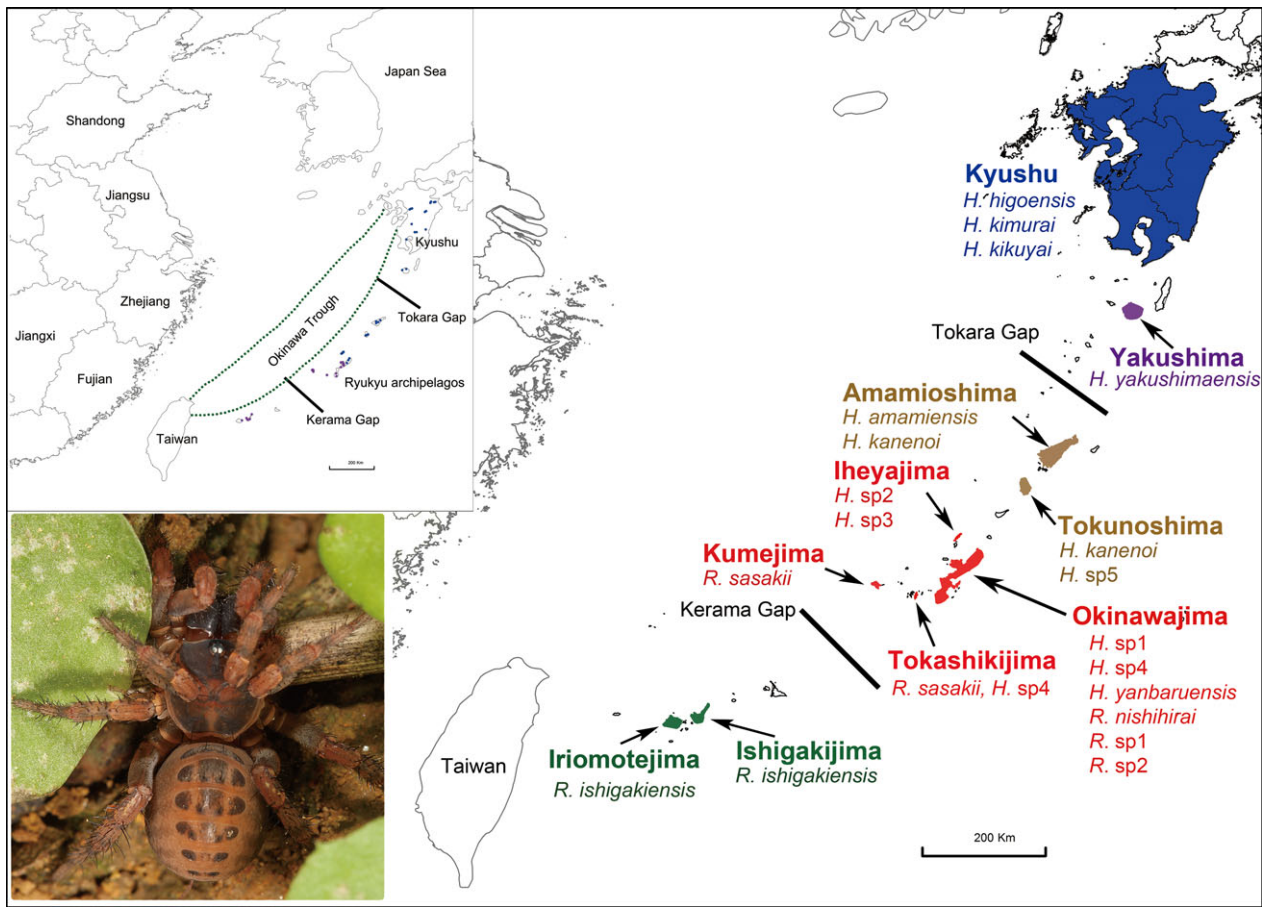
Figure 1 The study area and the sampled species of *Heptathela* and *Ryuthela* on East Asian margin (EAM) islands. We divided EAM into six discrete geographical areas: Kyushu (blue); the northern Ryukyus (Yakushima Island; purple); the Amami group of the central Ryukyus (Amamioshima and Tokunoshima; brown); the Okinawa group of the central Ryukyus (Iheyajima, Okinawajima, Kumejima and Tokashikijima; red); the southern Ryukyus (Ishigakijima and Iriomotejima; green); and east Asian mainland (area not coloured). Black bars indicate two geological boundaries, the Tokara Gap and the Kerama Gap, both corresponding to major past rivers. The inset map shows geographical places and collection localities; the inset image shows *Ryuthela sasakii*.

the five clearly defined areas within EAM islands (Fig. 1): three species of *Heptathela* are distributed in Kyushu, one in the northern, and eight in central Ryukyus (five undescribed); four species of *Ryuthela* (two undescribed) are found in the central and one species in the southern Ryukyus. No liphistiids are found in Taiwan or on the Japanese islands north of Kyushu.

Our previous study (Xu *et al.*, 2015a) suggested a vicariant origin of the EAM island liphistiid genera, but how geological events might have shaped the diversification and distribution of EAM island liphistiids at a species and population level remains unexplored. We thus explore pre-Pleistocene geological events shaping the diversification and distribution of EAM island liphistiids by testing biogeographical hypotheses and predictions outlined in Table 1. We sampled *Heptathela* and *Ryuthela* over their EAM island range, obtained data from mitochondrial and nuclear markers, and performed phylogenetic, phylogeographical, population genetic and historical demographic analyses to elucidate their origin, evolutionary history and divergence times.

MATERIALS AND METHODS

Taxonomic and population sampling

Our sampling at 58 localities across Kyushu Island and Ryukyu archipelago secured 12 species of *Heptathela* and five species of *Ryuthela* (see Table S1 in Appendix S1 Supporting Information). For phylogenetic tests, the 17 ingroup species were represented by one or two specimens from each site, totalling 115 specimens. A second tier of analyses were conducted including more intensive taxon sampling to assess genetic diversity and demographical history of the *Heptathela* and *Ryuthela* populations (for detailed information see Appendix S1).

Phylogenetic inference

Standard molecular protocols followed Xu *et al.* (2015a). We targeted fragments of two mitochondrial genes: cytochrome c oxidase subunit 1 (CO1) and 16S rRNA (16S), and three

Table 1 Biogeographical hypotheses and predictions about the origin and diversification within the East Asian Margin (EAM) island species of two genera, *Heptathela* and *Ryuthela*, inferred on the basis of the major pre-Pleistocene geological events within EAM. Vicariance hypotheses are supported when relationships and timing of splits match geological events; alternative hypotheses, including dispersal post-dating barrier formation, must be adopted when timing of diversification does not match tested vicariant events; note that dispersal events do not rule out vicariant past; tmrca: the most recent common ancestor.

Divergence between and within lineages	Vicariance event	Vicariance		Alternative	
		Hypothesis	Prediction	Hypothesis	Prediction
1 Between Asian continent and tmrca of *Heptathela+Ryuthela*	**Event 1:** Japan Sea opening (Kizaki & Oshiro, 1977; Otofuji *et al.*, 1985, 1991)	**H₁ₐ:** A sister group relationship between Asian continental species and tmrca of *Heptathela+Ryuthela* in EAM islands as a result of Event 1	Divergence timing of tmrca of *Heptathela+Ryuthela* coincides with timing of Event 1 at 23–15 Ma	**H₁ᵦ:** *Heptathela+Ryuthela* split prior to, or subsequent to EAM islands split from mainland; the latter implying dispersal	The crown age of *Heptathela+Ryuthela* pre- or post-dates Japan Sea opening, i.e., > 23 Ma or < 15 Ma
2 Within Okinawa Island Group lineages	**Event 2:** Formation of Okinawa Trough (Kimura, 2000; Kimura, 2003; Sibuet & Hsu, 2004)	**H₂ₐ:** A sister group relationship between Okinawa Island Group species and all remaining species in Asian continent and other EAM islands except Okinawa Island Group species as a result of Event 2	Divergence timing of Okinawa Island Group lineages coincides with timing of Event 2 at 10–6 Ma	**H₂ᵦ:** *Heptathela+Ryuthela* lineages in the Okinawa Island Group show ages that are inconsistent with vicariance; ancestral distribution preceding Event 2 would be consistent with 'out of Okinawa' scenario	Okinawa Island Group species pre-date 10 Ma (Okinawa as centre of origin), or post-date 6 Ma (dispersal)
3 Between Asia continent+Central Ryukyus+Northern Ryukyus+Kyushu and Southern Ryukyus	**Event 2a:** Isolation of Southern Ryukyus (Ujiié *et al*, 1991; Ujiié & Nakamura, 1996)	**H₃ₐ:** A sister group relationship between Southern Ryukyus species and all remaining areas within EAM as a result of Event 2a	Southern Ryukyus lineages should be ancient; divergence timing coincides with timing of Event 2a at 23–5.3 Ma	**H₃ᵦ:** Diversification of *Heptathela* and/or *Ryuthela* in the Southern Ryukyus is inconsistent with timing of vicariance	Diversification of lineages between Southern Ryukyus and all remaining areas within EAM pre-dates 23 Ma or post-dates 5.3 Ma
4 Between *Heptathela* and *Ryuthela*	**Event 2:** Formation of Okinawa Trough (Kimura, 2000; Kimura, 2003; Sibuet & Hsu, 2004)	**H₄ₐ:** A sister group relationship between *Heptathela* in an Ryukyus Island Group(s) and *Ryuthela* in the other Ryukyus Island Group(s) as a result of Event 2	Divergence timing between the two lineages within the Ryukyus coincides with timing of Event 2 at 10–6 Ma	**H₄ᵦ:** *Heptathela* and *Ryuthela* lineages in the Ryukyus show ages that are inconsistent with vicariance; ancestral distribution preceding Event 2 would be consistent with 'out of Ryukyus' scenario	*Heptathela* and *Ryuthela* divergences pre-date 10 Ma (Ryukyus as centre of origin), or post-date 6 Ma (dispersal)
5 Between Southern Ryukyus and Central Ryukyus	**Event 3:** Formation of Kerama Gap (Hikida & Ota, 1997; Ota, 1998)	**H₅ₐ:** A sister group relationship between Southern Ryukyus species and Central Ryukyus species was the result of Event 3	Divergence timing between the two lineages coincides with timing of Event 3 at 10–1.5 Ma	**H₅ᵦ:** Timing of divergence between Southern Ryukyus and Central Ryukyus does not coincide with Event 3	Southern and Central Ryukyus species are not sister lineages; and/or their divergence pre-dates 10 Ma, or post-dates 1.5 Ma

Table 1 Continued

	Divergence between and within lineages	Vicariance event	Vicariance		Alternative	
			Hypothesis	Prediction	Hypothesis	Prediction
6	Between Kyushu+Northern Ryukyus and Central Ryukyus	**Event 4:** Formation of Tokara Gap (Hikida & Ota, 1997; Ota, 1998)	H_{6a}: A sister group relationship between Kyushu+Northern Ryukyus and Central Ryukyus species as a result of Event 4	Divergence between the two lineages coincides with timing of Event 4 at 10–1.5 Ma	H_{6b}: Divergence of lineages in the Kyushu+Northern Ryukyus and Central Ryukyus does not coincide with Event 4	Kyushu+Northern Ryukyus species are not sister to the Central Ryukyus species; and/or divergences pre-date 10 Ma, or post-date 1.5 Ma
7	Between Kyushu and Northern Ryukyus	**Event 5:** Separation of Northern Ryukyus from Kyushu (Otsuka & Takahashi, 2000; Kimura, 2003)	H_{7a}: A sister group relationship between Kyushu and Northern Ryukyus species as a result of Event 5	Divergence timing between the two lineages coincides with timing of Event 5 at 1.3–0.2 Ma	H_{7b}: Diversification of lineages in Kyushu and Northern Ryukyus does not coincide with Event 5	Kyushu species are not sister to the Northern Ryukyus species; and/or divergences pre-date 1.3 Ma, or post-date 0.2 Ma
8	Between Amami Island Group and Okinawa Island Group	**Event 6:** Formation of the strait between Amami Island Group and Okinawa Island Group (Kizaki & Oshiro, 1977, 1980; Ota, 1998)	H_{8a}: A sister group relationship between the Amami and the Okinawa Island Group species as a result of Event 6	Divergence timing between the two lineages coincides with timing of Event 6 at 1.5–0.1 Ma	H_{8b}: Diversification of lineages in the Okinawa and the Amami Island Groups does not coincide with Event 6	Lineages in the Amami and the Okinawa Island Groups are not sister; and/or divergences pre-date 1.5 Ma, or post-date 0.1 Ma
9	Between Iheyajima and Okinawajima	**Event 7:** Separation of Iheyajima from Okinawajima (Honda et al., 2014)	H_{9a}: A sister group relationship between Iheyajima and Okinawajima species as a result of Event 7	Divergence timing between the two lineages coincides with timing of Event 7 at 1.8–0.01 Ma	H_{9b}: Diversification of lineages in Iheyajima and Okinawajima does not coincide with Event 7	Iheyajima and Okinawajima species are not sister; and/or divergences pre-date 1.8 Ma

nuclear loci: 28S rRNA (28S), histone 3 (H3) and internal transcribed spacer 2 (ITS2). We analysed the full data set based on two partition schemes: (1) the unlinked models for each gene (full gene partition scheme, NP); (2) the unlinked models for each gene and the unlinked first, second and third codon positions in CO1 (codon partition scheme, P). We treated gaps as missing data and inferred phylogenies using maximum likelihood (ML) in Garli 2.01 (Zwickl, 2006) and Bayesian inference (BI) analyses in MrBayes 3.2.1 (Ronquist *et al.*, 2012) (for details see Appendix S1).

Divergence dating

To estimate divergence times, we used fossil and biogeographical calibrations. The recently described *Cretaceothele lata* Wunderlich, 2015 from the Middle Cretaceous of Myanmar (Wunderlich, 2015) can only be attributed to the liphistiid stem due to uncertain cheliceral dentition. We constrained its age to the minimum 100 Ma (Xu *et al.*, 2015a). The opening of Japan Sea that split the EAM islands from Asia around 23–15 Ma (Kizaki & Oshiro, 1977, 1980; Otofuji *et al.*, 1985, 1991) was used to calibrate the split of clades containing the EAM island species, and the Asian continental species respectively. We used a normally distributed calibration prior, with a mean of 19 Ma and standard deviation of 2.4 Ma, instead of using the minimum bound at 15 Ma, based on both the geological event, the opening of Japan Sea, and the estimated divergence times of these lineages in our early study (Xu *et al.*, 2015a). We also used mitochondrial and nuclear substitution rates as described in our earlier study (Xu *et al.*, 2015a), and analysed the complete data set in beast 1.8.0 (Drummond *et al.*, 2012) using the relaxed uncorrelated lognormal molecular clock (Drummond *et al.*, 2006) and a Yule speciation model (see Appendix S1 for details).

Biogeographical reconstruction

We defined six discrete geographical areas (Fig. 1) based on plate-tectonic histories (e.g., Otofuji *et al.*, 1985; Tada, 1994; Ren *et al.*, 2002), as well as distributions and phylogenetic structure of liphistiid spiders (Xu *et al.*, 2015a): (1) Kyushu; (2) the northern Ryukyus (i.e. Yakushima Island in this study); (3) the Amami group of the central Ryukyus (i.e. Amamioshima and Tokunoshima in this study); (4) the Okinawa group of the central Ryukyus, including Iheyajima, Okinawajima, Kumejima and Tokashikijima; (5) the southern Ryukyus, including Ishigakijima and Iriomotejima; (6) mainland Asia including Hainan island.

To reconstruct ancestral distributions and to infer biogeographical events we used ultrametric time-calibrated trees from beast pruned for all but one outgroup terminal, and ran three alternative analyses implemented in rasp 3.0 (Yu *et al.*, 2010, 2014): (1) Bayesian binary MCMC analysis (BBM, Nylander *et al.*, 2008; Yu *et al.*, 2010); (2) statistical dispersal–vicariance analysis (S-DIVA, Yu *et al.*, 2010, 2014); and (3) dispersal-extinction-cladogenesis analysis (DEC, Ree

& Smith, 2008). We considered these three methods simultaneously to weigh the relative importance of vicariance, dispersal and extinction (see Appendix S1 for details).

Population genetic analyses

We used CO1 to calculate the number of haplotypes (h), haplotype diversity (Hd) and nucleotide diversity values (π) using DnaSP 5 (Librado & Rozas, 2009) (see Appendix S1). While using only maternally inherited mtDNA for population genetic analyses may have potential shortcomings (Edwards & Bensch, 2009), the lineages evaluated here are phylogenetically strongly supported by multilocus data (Xu *et al.*, 2015a). MtDNA is also widely used in phylogeographical studies of other spiders with lifestyles comparable to liphistiids (Hamilton *et al.*, 2011; Graham *et al.*, 2013, 2015).

Haplotype network analyses

To examine CO1 haplotype diversity in 17 species of *Heptathela* and *Ryuthela* we constructed parsimony haplotype networks in tcs 1.21 (Clement *et al.*, 2000) with a 95% connection limit. As this threshold does not necessarily correspond to species boundaries (Xu *et al.*, 2015c), we set the connection limit to 60 steps.

Historical demographical analyses

To assess potential historical population size changes, we conducted two tests of neutrality in CO1 data, the Tajima'D (Tajima, 1989) and Fu's Fs (Fu, 1997), and mismatch distribution analysis for each population and species (Rogers & Harpending, 1992; Schneider & Excoffier, 1999) as implemented in arlequin 3.5 (Excoffier & Lischer, 2010) (see Appendix S1).

RESULTS

Phylogenetic inference

The full, concatenated and aligned five-gene phylogenetic matrix included 125 sequences and 2686 sites (detailed sequence data in Table S1, Appendix S1). Bayesian and maximum likelihood analyses produced consistent topologies, and confirmed the monophyly of *Heptathela* + *Ryuthela*, endemic to EAM islands, and its sister relation with a Hainan clade (*Qiongthela*) to the exclusion of all continental species (Fig. 2).

All analyses further robustly support the monophyly of *Heptathela* and *Ryuthela* (Fig. 2). *Heptathela* contains a clade native to Kyushu and Yakushima Island and a clade with eight species from the central Ryukyus, the Amami group and Okinawa group (Fig. 2). Within the central Ryukyus, one clade groups the species from the Amami group and from northern Okinawa (*H.* sp1), while another clade contains only species from Okinawa group (Fig. 2). Within
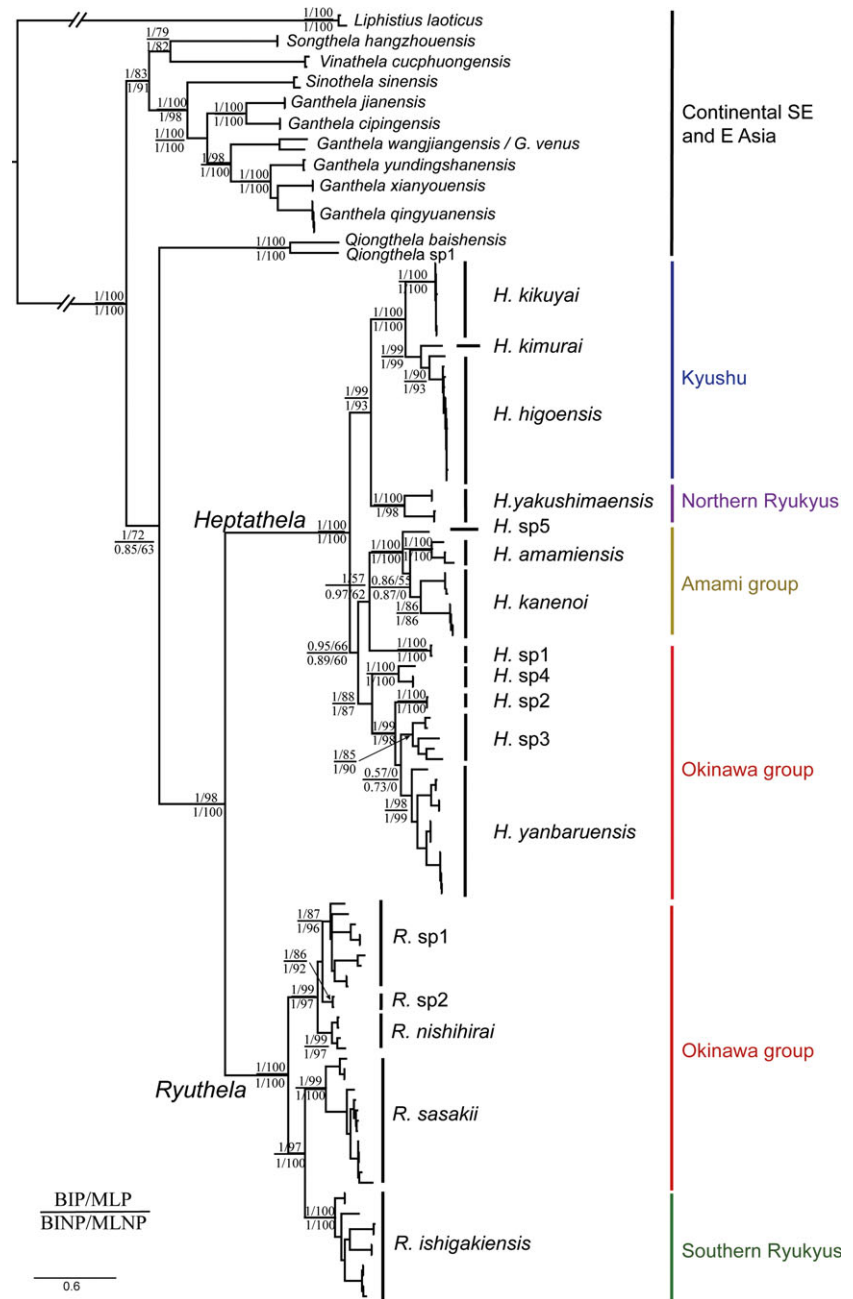
Figure 2 Summary phylogenetic results from Bayesian inference (BI) and maximum likelihood (ML) analyses of different partition schemes of the primitively segmented spider genera *Heptathela* and *Ryuthela*. The topology is from the Bayesian analysis of the full and partitioned matrix (see methods). The numbers indicate branch supports from BI (posterior probability) and ML (bootstrap).

Ryuthela, a clade with the species from Okinawa group [*R. nishihirai* (Haupt, 1979), *R.* sp1-2] is sister to a clade containing a species from Okinawa group and a species from the southern Ryukyus (Fig. 2).

Divergence dating

Divergence timing results and chronogram topologies resemble the phylogeny (Figs 2 & 3). The mean estimate of the age of the most recent common ancestor (tmrca) for the EAM island clade *Heptathela* + *Ryuthela* placed it in the early Miocene, 22.4 Ma (95% HPD: 26.6–18.4 Ma). The genera *Heptathela* (tmrca: 14.2 Ma; 18.0–10.7 Ma) and *Ryuthela* (12.9 Ma; 17.8–8.7 Ma) originated during the middle Mio-

cene (Fig. 3; Table S3 in Appendix S2). For the estimated ages of the internal nodes of *Heptathela* and *Ryuthela* see Fig. 3 and Table S3.

The species level divergence times in *Heptathela* range from 0.3 Ma (*H.* sp2) to 5.3 Ma (*H. yakushimaensis* Ono, 1998), with seven species dating to Pliocene and others to Pleistocene (Fig. 3; Table S3). In *Ryuthela*, four species date to late Miocene and Pliocene (6.0–2.9 Ma) and one to Pleistocene (0.6 Ma) (Fig. 3; Table S3).

Biogeographical reconstruction

Figure 4 recaps the reconstructed ancestral areas using three different methods (BBM, S-DIVA, DEC; Fig. S1 and
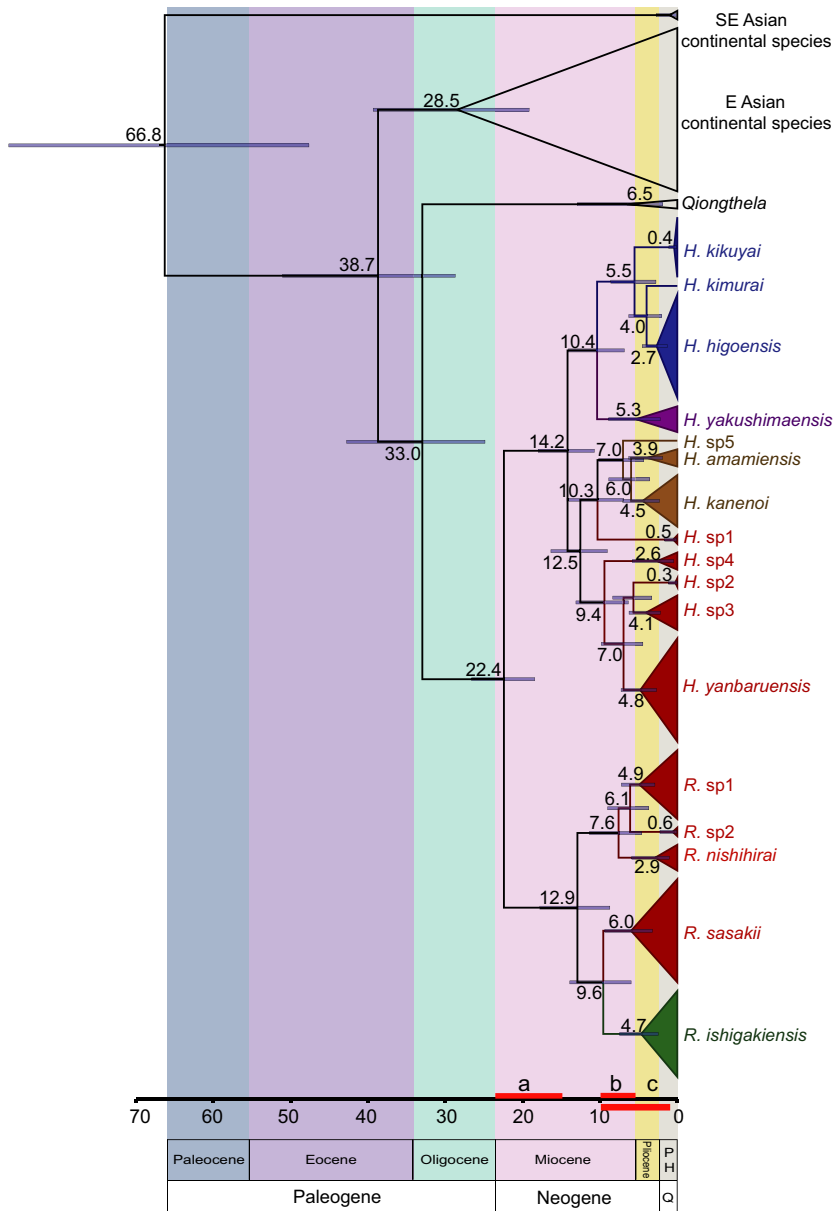
Figure 3 The chronogram with inferred node age in million years (Ma) from BEAST analyses and with 95% confidence intervals (nodal bars). Taxa are colour coded according to areas defined in Fig. 1. Horizontal red bars mark geological events affecting *Heptathela* and *Ryuthela* biogeographical histories. (a) Japanese islands separated from East Asian continent; (b) Ryukyu Archipelago separated from East Asian continent; (c) the formation of Tokara and Kerama Gaps.

Table S4 in Appendix S2). The EAM island clade *Heptathela* + *Ryuthela* (node 2) origin is in Okinawa group according to BBM, or in any island area except for southern Ryukyus according to S-DIVA and DEC. The results are similar for *Heptathela* (node 3), but all methods postulate at least one vicariant event within the clade. In *Ryuthela* (node 7), the reconstructed ancestral area is predominantly the Okinawa group, with DEC method also allowing for the southern Ryukyus and two of the three methods implying a dispersal event. At the lower phylogenetic level within *Ryuthela* (node 8), however, the shift from the Okinawa group to southern Ryukyus is explained by vicariance. The reconstructed ancestral area of the *Heptathela* node 6 uniting species from both the Amami group and Okinawa group is Okinawa group (or the Amami group/Okinawa group) with a postulated vicariance event according to S-DIVA and DEC, but with both vicariance and dispersal according to BBM. While ancestral

areas for certain other nodes are also resolved equivocally (e.g. node 4), most shallow nodes are resolved unambiguously (Fig. 4).

Population genetic analyses

We obtained partial CO1 sequence data (680 bp) for several individuals of *H. amamiensis* Haupt, 1983 (8 individuals), *H. higoensis* Haupt, 1983 (40), *H. kikuyai* Ono, 1998 (37), *H. kanenoi* Ono, 1996 (24), *H. kimurai* (Kishida, 1920) (10), *H. yakushimaensis* (9), *H. yanbaruensis* Haupt, 1983 (33), *H.* sp1 (10), *H.* sp2 (9), *H.* sp3 (29), *H.* sp4 (6), *H.* sp5 (6), *R. ishigakiensis* Haupt, 1983 (30), *R. nishihirai* (14), *R. sasakii* Ono, 1997 (82), *R.* sp1 (27) and *R.* sp2 (5).

The within-population genetic diversity varied among species at an island group scale (Table S5 in Appendix S2). Overall, genetic diversity measured as number of haplotypes
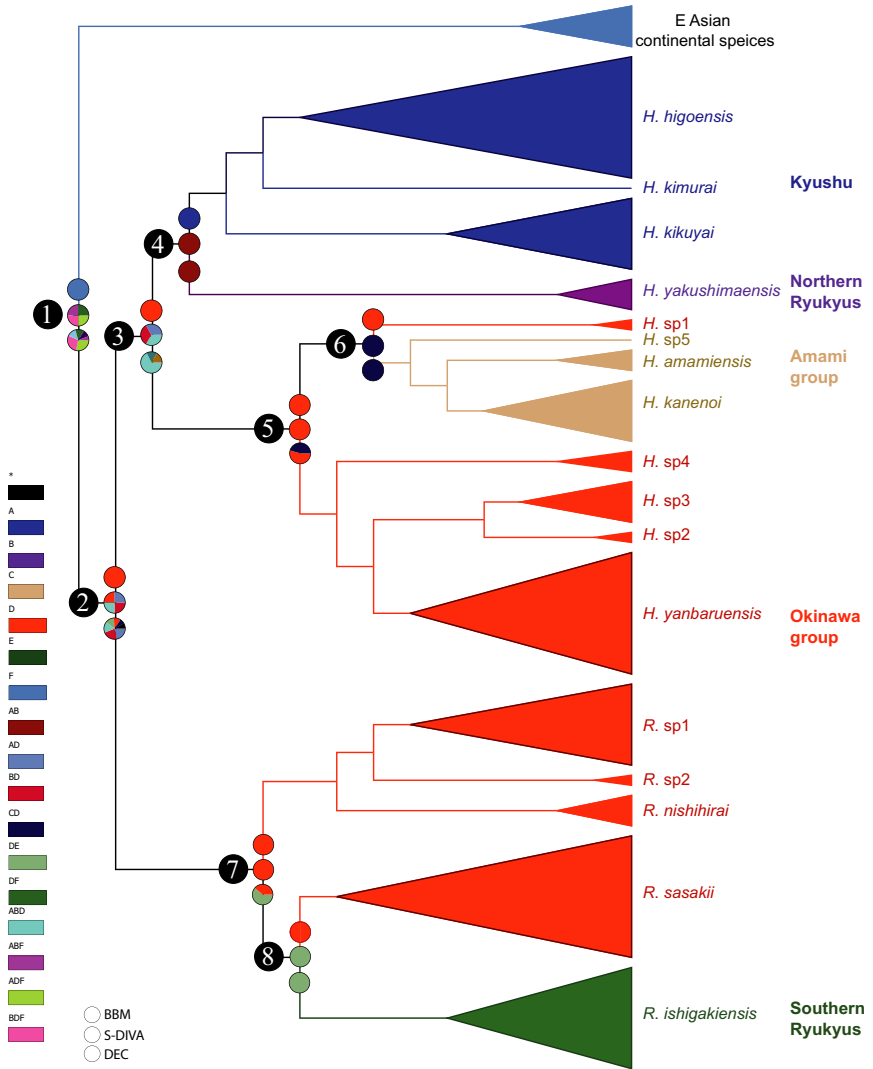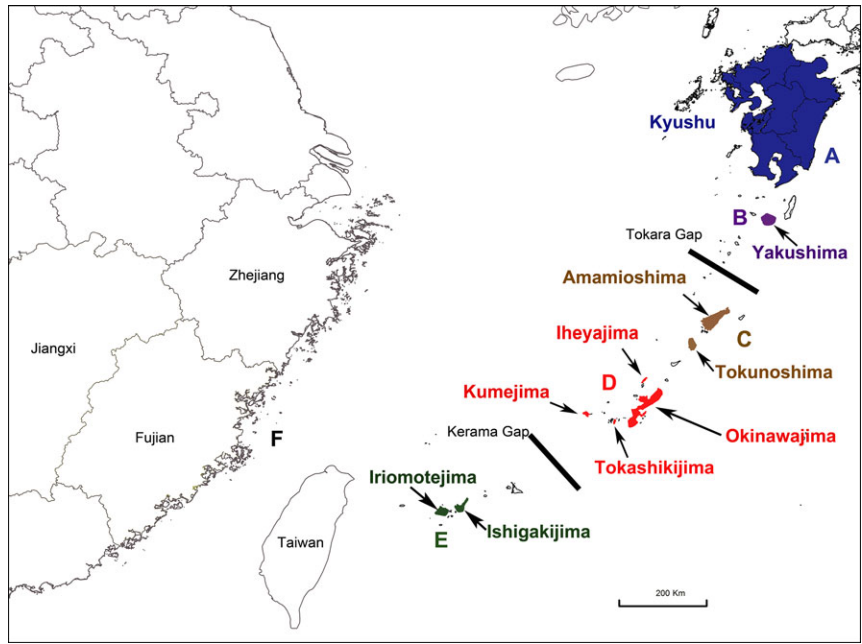
Figure 4 Ancestral area reconstructions of *Heptathela* and *Ryuthela* on East Asian margin (EAM) islands. Geographical areas in the legend correspond to letters in the upper map. Black numbered circles represent the major nodes matching those in Table S4. Pie charts at main nodes 1–8 show the most likely ancestral areas as reconstructed by BBM, S-DIVA and DEC (see methods).

(*h*), haplotype diversity (*Hd*), and nucleotide diversity (π), was relatively high in the central and southern Ryukyus, and relatively low in Kyushu and the northern Ryukyus (Yakushima) (Table S5). *Heptathela* species showed relatively lower genetic diversities compared to *Ryuthela* species. Within *Heptathela*, *H. higoensis*, *H. kikuyai* and *H. kimurai* in Kyushu had low population genetic diversity compared with *H. kanenoi*, *H. yanbaruensis* and *H. sp3* in the central Ryukyus, if species with one sampled population were excluded. In *Ryuthela*, *R. nishihirai* and *R. sp1* in Okinawajima showed relatively low genetic diversity as compared to *R. ishigakiensis* in the southern Ryukyus and *R. sasakii* on Kumejima/Tokashikijima.

Haplotype network analyses

The statistical parsimony networks for the populations of 15 species connected all conspecific populations when setting the connection limit to 95% in the case of *H. higoensis*, *H. kikuyai*, *H. kimurai*, *H. sp1*, *H. sp5*, and *R. sp2* (Fig. 5). In all the remaining species (Figs 5 & 6), the networks were connected by fixing the connection limit to 60 steps. This network analysis postulated relatively few substitutions among haplotypes in Kyushu *Heptathela* (Fig. 5a), in *H. sp5* confined to Tokunoshima and in *H. sp1* from northern Okinawa (Fig. 5b). Most other *Heptathela*, however, particularly those inhabiting more than a single island, showed much more complicated network structures that implied numerous substitutions between haplotypes. With the exception of *R. sp2* from central Okinawa, all other *Ryuthela* species showed complicated networks of haplotypes, postulating up to 50 substitutions within (*R. ishigakiensis*) and up to 51 substitutions between islands (Fig. 6).

Historical demographical analyses

For the 15 analysed species with several haplotypes, the values of Tajima's *D* and Fu's *Fs* test of neutrality for all populations and each species were non-significant (Table S5), suggesting that past population sizes were stable. The results of mismatch distribution under the spatial expansion models was non-significant for all populations (*P* values for the sum of squared deviations (SSD) and Harpending's raggedness index (HRI) > 0.05; Table S5), while the results under the population expansion model were significant in *H. kanenoi*, *H. yanbaruensis*, *H. sp3*, *R. ishigakiensis*, *R. nishihirai* and *R. sp1* (Table S5).

DISCUSSION

As a test of historical geological reconstructions, we examined phylogenetic, biogeographical and population genetic patterns of an endemic spider clade on the EAM islands, *Heptathela* + *Ryuthela,* whose natural history suggests them to be dispersal limited. Consistent with our earlier study (Xu *et al.*, 2015a) that revealed a vicariant origin of the EAM

liphistiids at the genus level, the present study confirms predominantly vicariant phylogeographical patterns at the species and population levels. Congruent with our hypotheses, *Heptathela* and *Ryuthela* cladogenetic histories were most likely shaped by the seven Cenozoic major tectonic events on EAM (Table 1): (1) the openings of the Japan Sea and (2) the Okinawa Trough (tmrca of both *Heptathela* and *Ryuthela* split from Asian continental species), (3) the formation of the Kerama (*R. ishigakiensis* split from *R. sasakii*) and (4) Tokara gaps (Kyushu and northern Ryukyus *Heptathela* split from central Ryukyus *Heptathela*), (5) the separation of the Amami group from the Okinawa group (*H. sp1* splits from Amami group species) and (6) the separation of the northern Ryukyus from Kyushu (*H. yakushimaensis* split from Kyushu species), as well as (7) the isolation of Iheyajima from the Okinawa group (*H. sp2* + *H. sp3* split from *H. yanbaruensis*).

Consistent with H_{1a}, tmrca of *Heptathela* + *Ryuthela* (26.6–18.4 Ma; Fig. 3, Table S3) coincides with EAM island separation from Asia (Ujiié, 1990), although this is not surprising given our calibration scheme. The origin of *Heptathela* + *Ryuthela* recovered by ancestral area reconstruction is also congruent with the vicariance hypothesis: their ancestors were widespread and had already speciated across the areas that subsequently formed the EAM islands, with an exception of southern Ryukyus (Figs 3, 4 & S1, Table S4). Phylogenetic and divergence dating analyses recovered the highest species richness of ancient lineages of *Heptathela* and *Ryuthela* in the Okinawa group, which is also where BBM reconstructed their ancestral distributions (Fig. 4). These results are consistent with H_{2b} and H_{4b} (Table 1) postulating Okinawa group (Area D in Fig. 4) as the centre of origin of EAM island liphistiids. Both genera might have originated in palaeo-Okinawa, then expanded their ranges northwards (*Heptathela*) and southwards (*Ryuthela*) before the island fragmentation.

Miocene origins of *Heptathela* (18.0–10.7 Ma) and *Ryuthela* (17.8–8.7 Ma) are vicariant, coinciding with the opening of the Japan Sea and the Okinawa Trough (references above). These are consistent with H_{1a} and H_{4b} (Table 1). The split of *R. sasakii* and *R. ishigakiensis* (13.9–6.0 Ma, Fig. 3, Table S3) coincided with the emergence of the Kerama Gap (references above), corroborating H_{5a} (Table 1). This gap likely acted as a geographical barrier judging from restricted gene flow between these lineages during Pleistocene (Figs 3 & 6). Our results lend support to Ota's (1998) hypothesis that central Ryukyus were isolated relatively early from the other island groups, and contradict H_{3b} (Table 1) that the southern Ryukyu islands were separated from the Asian continent prior to the remaining Ryukyus (references above).

Our results also support vicariant speciation following the formation of other physical barriers, but the evidence for vicariant history of *Heptathela* between middle Miocene and Pleistocene is inconclusive. The first divergence of the central Ryukyus *Heptathela* lineages from the northern Ryukyus plus
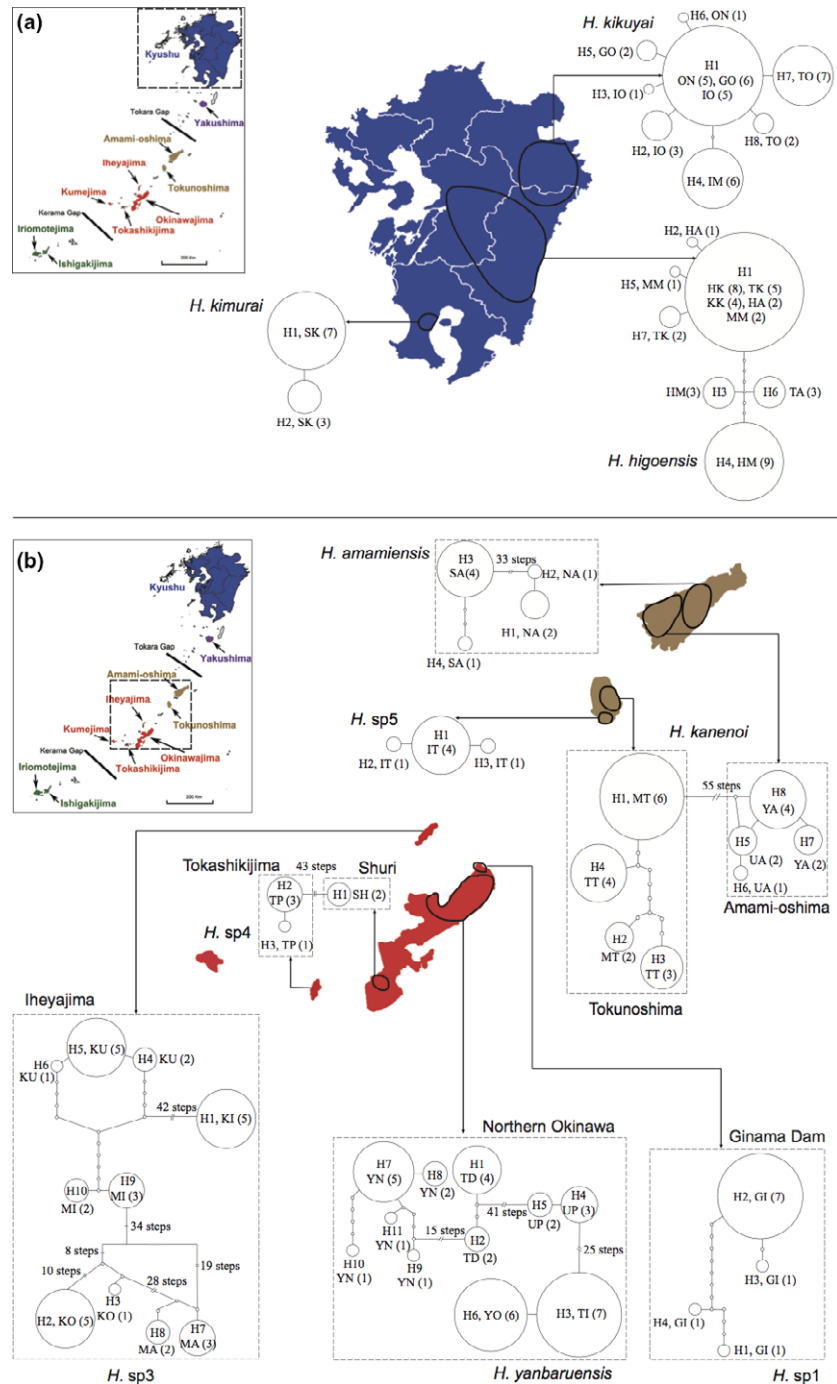
Figure 5 Statistical parsimony networks depicting relationships among CO1 haplotypes in *Heptathela* species from: (a) Kyushu; and (b) the Amami group of the central Ryukyus (Amamioshima and Tokunashima) and the Okinawa group of the central Ryukyus (Iheyajima, Okinawajima and Tokashikijima). The inset map shows the study area (colour-coded as in Fig. 1) on East Asian margin (EAM) islands. The size of each open circle indicates haplotype frequency. Open dots on lines between haplotypes indicate 1 mutation step. Numbers preceded by "H" indicate haplotype number. Two capital letters, corresponding to codes in Table S5, indicating the population are followed by sample size in parentheses. Dashed lines enclose haplotype networks confined to an island or region.

Kyushu *Heptathela* lineages was estimated at 18.0–10.7 Ma (Fig. 3 and Table S3), and the biogeographical reconstructions indicated a vicariant divergence between these clades. While relationships support H_{6a}, the divergence timing predates the formation of the Tokara Gap. This implies either earlier unknown vicariant events, or Tokara Gap's earlier emergence than suggested by literature (Otsuka & Takahashi, 2000; Kimura, 2003). In addition, although relationships among *Heptathela* species in the northern Ryukyus and Kyushu (Fig. 3) corroborate H_{7a} (Table 1), the middle-late Miocene (14.1–6.9 Ma) diversification pre-dates the

understood Pleistocene strait formation (1.3–0.2 Ma) (references above). Furthermore, the divergence within *Heptathela* of central Ryukyus (14.3–6.9 Ma) much predated the split of the Amami group from the Okinawa group (1.5–1.0 Ma: Kizaki & Oshiro, 1977, 1980; 0.8–0.1 Ma: Ota, 1998), a pattern resembling regional amphibian species distributions (Matsui *et al.*, 2005; Tominaga *et al.*, 2010, 2015; Honda *et al.*, 2012). Although this result is topologically consistent, these divergencies are inconsistent with those predicted by H_{8a} (Table 1). Finally, Iheyajima species (*H.* sp2, *H.* sp3) are nested within Okinawajima species, and split from *H.*
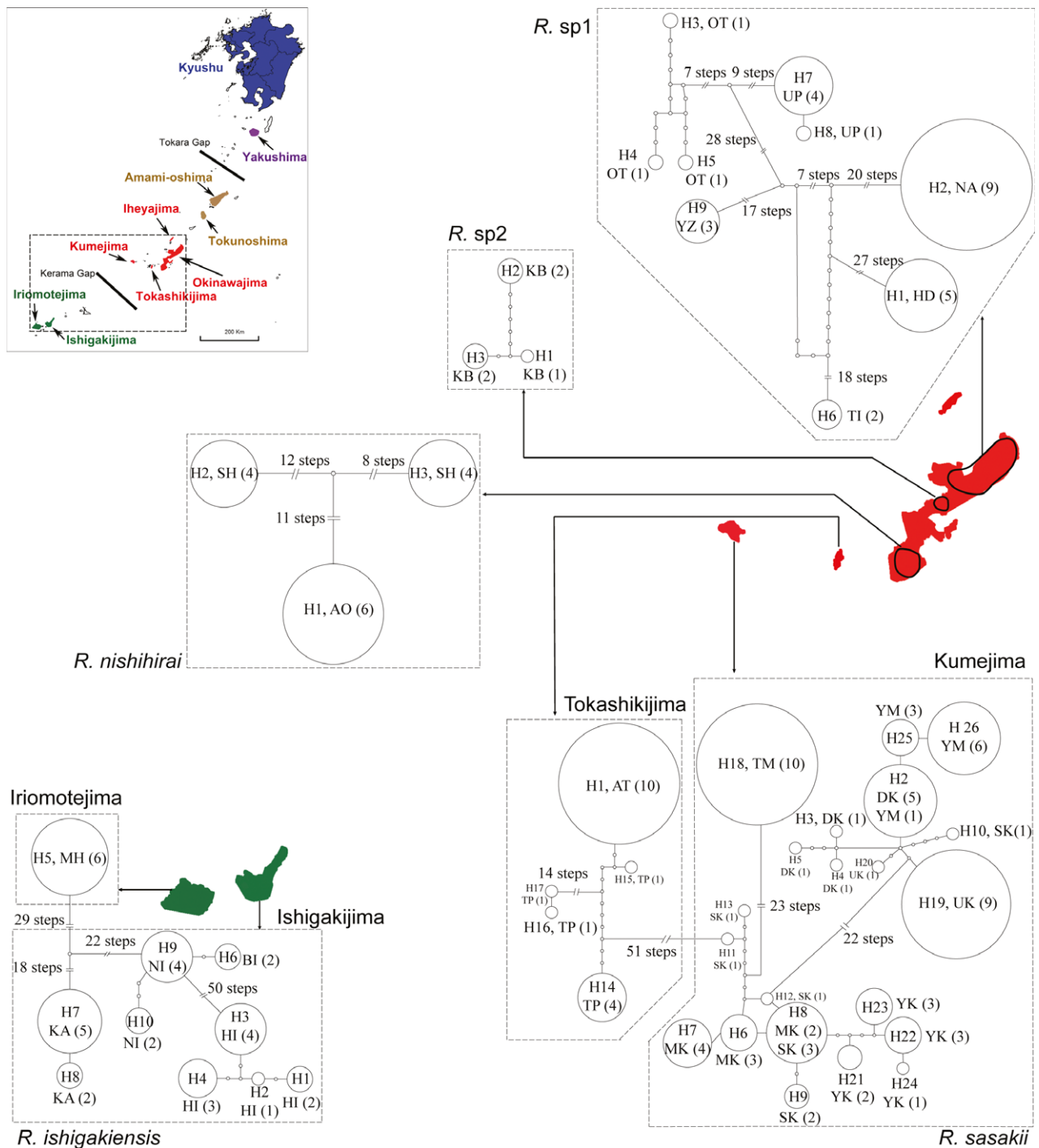
Figure 6 Statistical parsimony networks depicting relationships among CO1 haplotypes in *Ryuthela* species from EAM. Abbreviations and legends as in Figs 1 & 5.

yanbaruensis at 9.8–4.5 Ma, long before the separation of Iheyajima from Okinawajima at 1.8–0.01 Ma (Honda *et al.*, 2014), an incongruent scenario with H_{9a} (Table 1). This suggests that the divergence between Iheyajima and Okinawajima lineages occurred in the absence of an intervening strait. Combined, these results agree with other studies of EAM islands (e.g. Tominaga *et al.*, 2010, 2015; Honda *et al.*, 2012) suggesting that vicariant histories may result

from barriers that may be older than those highlighted in Table 1, and yet unknown.

The paraphyly of Okinawa group *Heptathela* lineages (Figs 2 & 3) suggests a possible secondary range expansion of *Heptathela* from the Okinawa group to the Amami group after initial separation of the two clades (Figs 2 & 3). The paraphyly of Okinawa group *Ryuthela* (Figs 2 & 3) indicates that *Ryuthela* might have expanded into south-west islands

of the Okinawa group and the southern Ryukyus before the formation of the Kerama Gap, which split *R. ishigakiensis* from *R. sasakii* (Figs 2 & 3). The divergence of *Heptathela* in central Ryukyus and of *Ryuthela* in central and southern Ryukyus recovered by ancestral area reconstruction is incongruent with the main vicariant hypothesis (Table 1; Fig. 4; Table S4).

Taiwan only formed about 9–5 Ma (Sibuet & Hsu, 2004). Our results suggest that tmrca of EAM island liphistiids inhabited the land mass that is today south-western Japan before the islands split from the mainland around 22.4 Ma. Congruent with our earlier study on the global biogeography of liphistiids (Xu *et al.*, 2015a), liphistiid sedentary terrestrial life history and no over-water dispersal suggest that the Pleistocene land bridge connecting Taiwan with Ryukyus (Ota, 1998; Kimura, 2003) was too short-lived to allow potential colonization of Taiwan by slow moving liphistiids.

Heptathela and *Ryuthela* lineages on the EAM islands show robust phylogeographical patterns. This EAM island clade consists of five genetically distinct, geographically structured, and partially codistributed lineages (Figs 1–3). Both genera codistributed on the Okinawa group exhibit comparable population structures characterized by high genetic diversity (Table S5), deep splits among lineages (Fig. 3, Table S3), multiple isolated genetic lineages (Figs 2, 3, 5 & 6), and stable populations (Table S5), suggesting that these lineages diversified well before the Pleistocene glaciations and that their matching phylogeographical patterns have been shaped by late Cenozoic geological events, some of which are yet unknown. Deep genetic structuring is known in mygalomorph spiders that share phenotypic and ecological traits with liphistiids (Bond *et al.*, 2001; Opatova & Arnedo, 2014b). In contrast, the population genetic structures of *Heptathela* lineages of Kyushu and the Amami group exhibit lower genetic diversity reflected by high haplotype diversity and low nucleotide diversity indices (Table S5), simpler haplotype networks (Fig. 5), shallow splits among lineages (Fig. 3), and good fits of both sudden and spatial expansion models (Table S5). These contrasting phylogeographical patterns of liphistiid spiders in Ryukyus and Kyushu underline the different geological history of these island groups. It seems that population structures of the *Heptathela* lineages on Amami group and Kyushu may have been more affected by the Pleistocene glaciations compared with those lineages on Okinawa group. The Ryukyu islands have been isolated from Kyushu since 1.5 Ma (Kizaki & Oshiro, 1977), and have maintained a warmer and stable climate compared with the main islands judging from pollen records indicating the existence of broadleaved evergreen trees throughout the glacial periods (Aoki *et al.*, 2008). Although Kyushu was ice-free in Pleistocene, strong climatic fluctuations may have significantly affected *Heptathela* populations during that period. Consequently, the stable subtropical Ryukyus harbour more genetically diverse liphistiids compared with Kyushu where glacial events may have wiped out portions of the genetic diversity.

Results from network, population genetic and historical demographic analyses confirm that populations of *Heptathela* and *Ryuthela* lineages do not maintain gene flow within and among islands. These results indicate that in these dispersal-limited spiders, small scale geographical barriers such as mountains and rivers are sufficient to effectively preclude dispersal within islands. Similarly, among islands, even shallow oceanic barriers due to shifting sea level during the glacial periods may have isolated island populations by restricting gene flow. The results of neutrality tests indicate that these island species have had seemingly stable past populations. This may suggest that once populations are trapped on fragmented islands, their lack of over-water dispersal ability interrupts any inter-island gene flow, resulting in vicariant speciation, but populations of emerging species (albeit relatively small) do not suffer from bottleneck effects.

CONCLUSIONS

We provide a time-calibrated species level phylogeny of EAM island liphistiids aimed to reconstruct their biogeographical history and thereby test the historical geological reconstruction of the EAM islands. Natural history predicts primitively segmented spiders to be sedentary and dispersal-limited, and this hypothesis was supported by population genetic and historical demographical analyses, and the recovered predominant vicarance events. We identified some major geohistorical events, and postulated unknown ones, that resulted in diversification of insular liphistiid lineages through vicariance during the middle and late Miocene. Our results suggest that restricted gene flow following barrier formation is the dominant force governing the historical and current-day genetic boundaries among and within islands, which indicates the broad-scale impact of the pre-Pleistocene tectonic events on codistributed continental insular lineages. Together with a few well-known phylogenetically old terrestrial arachnid lineages such as harvestmen (Boyer *et al.*, 2007; Clouse & Giribet, 2010) and myglomorph spiders (Opatova & Arnedo, 2014a, b), liphistiids are a rare example of dispersal-limited organisms occupying continental islands where overwater dispersal is evidently absent. Hence, their phylogenetic history can be treated as independent tests of geological history.

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SUPPORTING INFORMATION

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

Appendix S1 Detailed descriptions of materials and methods.
Appendix S2 Result explanations.

BIOSKETCH

Xin Xu concluded her PhD study at the Centre for Behavioural Ecology and Evolution, College of Life Sciences, Hubei University. Her dissertation focused on the biogeography, systematics and evolution of the primitively segmented spiders.

Her PhD advisors, **Daiqin Li** and **Matjaž Kuntner** share research interest with **Fengxiang Liu, Jian Chen** and **Hirotsugu Ono** in spider systematics and evolution, and with **Ingi Agnarsson** in historic biogeography, focusing on vicariant evolutionary histories and on organismal traits that may affect their dispersal abilities.

Authors' contribution: X.X., D.L., M.K., H.O. and J.C designed the study. X.X., F.X.L., D.L. and H.O. performed the fieldwork. X.X. conducted the lab work. X.X., D.L. and M.K. analysed the data. X.X., D.L., M.K. and I.A. interpreted the results and wrote the paper.

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