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

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\textbf{ABSTRACT}

\textbf{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, \textit{Heptathela} and \textit{Ryuthela} (Liphistiidae), broadly codistributed across EAM islands. To test competing biogeographical hypotheses, we looked for geohistoric events that may have shaped their distributional patterns.

\textbf{Location} Kyushu and Ryukyus.

\textbf{Methods} We sampled 17 \textit{Heptathela} and \textit{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.

\textbf{Results} Phylogenetic origin of \textit{Heptathela} + \textit{Ryuthela} is estimated at 26.6–18.4 Ma (95\% HPD) when EAM islands became separated from the continent. The crown ages of \textit{Heptathela} (18.0–10.7 Ma) and \textit{Ryuthela} (17.8–8.7 Ma) coincide with the formation of Japan Sea and Okinawa Trough respectively. A split within \textit{Ryuthela} (13.9–6.0 Ma) coincides with the opening of the Kerama Gap, but a split within \textit{Heptathela} (18.0–10.7 Ma) pre-dates the formation of the Tokara Gap. Speciation within \textit{Heptathela} and \textit{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.

\textbf{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.

\textbf{Keywords} biogeography, Cenozoic, continental islands, dispersal, Kyushu, phylogeography, Ryukyus, vicariance
**INTRODUCTION**

Islands represent natural laboratories for studying biogeography and evolution because of their complex geological history, well-defined boundaries, limited gene flow and high endemcity (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 Ryuku islands from the continent in the Miocene (23–5.3 Ma), while the remaining Ryukyus were still part of Asia (Ujić *et al.*, 1991; Ujić & 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 disunite older patterns (Sanmartín & Ronquist, 2004; Upchuch, 2008; Sklenárová *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 *Ruthela* 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 *Ruthela* have endemic species each confined to one (very rarely two) of
the five clearly defined areas within EAM islands (Fig. 1): three species of \textit{Heptathela} are distributed in 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 \textit{Ryuthela} sasakii.

\textbf{Figure 1} The study area and the sampled species of \textit{Heptathela} and \textit{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 \textit{Ryuthela} sasakii.

\textbf{MATERIALS AND METHODS}

\textbf{Taxonomic and population sampling}

Our sampling at 58 localities across Kyushu Island and Ryukyu archipelago secured 12 species of \textit{Heptathela} and five species of \textit{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 \textit{Heptathela} and \textit{Ryuthela} populations (for detailed information see Appendix S1).

\textbf{Phylogenetic inference}

Standard molecular protocols followed Xu \textit{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.

<table>
<thead>
<tr>
<th>Divergence between and within lineages</th>
<th>Vicariance event</th>
<th>Vicariance Hypothesis</th>
<th>Prediction</th>
<th>Alternative Hypothesis</th>
<th>Prediction</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Between Asian continent and tmrca of <em>Heptathela</em> + <em>Ryuthela</em></td>
<td>Event 1: Japan Sea opening (Kizaki &amp; Oshiro, 1977; 1980; Otofuji <em>et al</em>., 1985, 1991)</td>
<td><strong>H1</strong>: A sister group relationship between Asian continental species and tmrca of <em>Heptathela</em> + <em>Ryuthela</em> in EAM islands as a result of Event 1</td>
<td>Divergence timing of tmrca of <em>Heptathela</em> + <em>Ryuthela</em> coincides with timing of Event 1 at 23–15 Ma</td>
<td><strong>H1</strong>: <em>Heptathela</em> + <em>Ryuthela</em> split prior to, or subsequent to EAM islands split from mainland; the latter implying dispersal</td>
<td>The crown age of <em>Heptathela</em> + <em>Ryuthela</em> pre- or post-dates Japan Sea opening, i.e., &gt; 23 Ma or &lt; 15 Ma</td>
</tr>
<tr>
<td>2 Within Okinawa Island Group lineages</td>
<td>Event 2: Formation of Okinawa Trough (Kimura, 2000; Kimura, 2003; Sibuet &amp; Hsu, 2004)</td>
<td><strong>H2</strong>: 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</td>
<td>Divergence timing of Okinawa Island Group lineages coincides with timing of Event 2 at 10–6 Ma</td>
<td><strong>H2</strong>: <em>Heptathela</em> + <em>Ryuthela</em> 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</td>
<td>Okinawa Island Group species pre-date 10 Ma (Okinawa as centre of origin), or post-date 6 Ma (dispersal)</td>
</tr>
<tr>
<td>3 Between Asian continent + Central Ryukyus + Northern Ryukyus + Kyushu and Southern Ryukyus</td>
<td>Event 2a: Isolation of Southern Ryukyus (Ujiie <em>et al</em>., 1991; Ujiie &amp; Nakamura, 1996)</td>
<td><strong>H3</strong>: A sister group relationship between Southern Ryukyus species and all remaining areas within EAM as a result of Event 2a</td>
<td>Divergence timing of Southern Ryukyus lineages should be ancient; divergence timing coincides with timing of Event 2a at 23–5.3 Ma</td>
<td><strong>H3</strong>: Diversification of <em>Heptathela</em> and/or <em>Ryuthela</em> in the Southern Ryukyus is inconsistent with timing of vicariance</td>
<td>Diversification of lineages between Southern Ryukyus and all remaining areas within EAM pre-dates 23 Ma or post-dates 5.3 Ma</td>
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<tr>
<td>4 Between <em>Heptathela</em> and <em>Ryuthela</em></td>
<td>Event 2: Formation of Okinawa Trough (Kimura, 2000; Kimura, 2003; Sibuet &amp; Hsu, 2004)</td>
<td><strong>H4</strong>: A sister group relationship between <em>Heptathela</em> in an Ryukyus Island Group (s) and <em>Ryuthela</em> in the other Ryukyus Island Group(s) as a result of Event 2</td>
<td>Divergence timing between the two lineages within the Ryukyus coincides with timing of Event 2 at 10–6 Ma</td>
<td><strong>H4</strong>: <em>Heptathela</em> and <em>Ryuthela</em> lineages in the Ryukyus show ages that are inconsistent with vicariance; ancestral distribution preceding Event 2 would be consistent with 'out of Ryukyus' scenario</td>
<td><em>Heptathela</em> and <em>Ryuthela</em> divergences pre-date 10 Ma (Ryukyus as centre of origin), or post-date 6 Ma (dispersal)</td>
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<td>5 Between Southern Ryukyus and Central Ryukyus</td>
<td>Event 3: Formation of Kerama Gap (Hikida &amp; Ota, 1997; Ota, 1998)</td>
<td><strong>H5</strong>: A sister group relationship between Southern Ryukyus species and Central Ryukyus species was the result of Event 3</td>
<td>Divergence timing between the two lineages coincides with timing of Event 3 at 10–1.5 Ma</td>
<td><strong>H5</strong>: Timing of divergence between Southern Ryukyus and Central Ryukyus does not coincide with Event 3</td>
<td>Southern and Central Ryukyus species are not sister lineages; and/or their divergence pre-dates 10 Ma, or post-dates 1.5 Ma</td>
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<tr>
<td>Divergence between and within lineages</td>
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<td>6 Between Kyushu+Northern Ryukyus and Central Ryukyus</td>
<td>Event 4: Formation of Tokara Gap (Hikida &amp; Ota, 1997; Ota, 1998)</td>
<td>$H_{6a}$: A sister group relationship between Kyushu+Northern Ryukyus and Central Ryukyus species as a result of Event 4</td>
<td>$H_{6b}$: Divergence of lineages in the Kyushu+Northern Ryukyus and Central Ryukyus does not coincide with Event 4</td>
<td>Divergence between the two lineages coincides with timing of Event 4 at 10–1.5 Ma</td>
<td>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</td>
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<td>7 Between Kyushu and Northern Ryukyus</td>
<td>Event 5: Separation of Northern Ryukyus from Kyushu (Otsuka &amp; Takahashi, 2000; Kimura, 2003)</td>
<td>$H_{7a}$: A sister group relationship between Kyushu and Northern Ryukyus species as a result of Event 5</td>
<td>$H_{7b}$: Diversification of lineages in Kyushu and Northern Ryukyus does not coincide with Event 5</td>
<td>Divergence timing between the two lineages coincides with timing of Event 5 at 1.3–0.2 Ma</td>
<td>Kyushu species are not sister to the Northern Ryukyus species; and/or divergences pre-date 1.3 Ma, or post-date 0.2 Ma</td>
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<td>8 Between Amami Island Group and Okinawa Island Group</td>
<td>Event 6: Formation of the strait between Amami Island Group and Okinawa Island Group (Kizaki &amp; Oshiro, 1977, 1980; Ota, 1998)</td>
<td>$H_{8a}$: A sister group relationship between the Amami and the Okinawa Island Group species as a result of Event 6</td>
<td>$H_{8b}$: Diversification of lineages in the Okinawa and the Amami Island Groups does not coincide with Event 6</td>
<td>Divergence timing between the two lineages coincides with timing of Event 6 at 1.5–0.1 Ma</td>
<td>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</td>
</tr>
<tr>
<td>9 Between Iheyajima and Okinawajima</td>
<td>Event 7: Separation of Iheyajima from Okinawajima (Honda et al., 2014)</td>
<td>$H_{9a}$: A sister group relationship between Iheyajima and Okinawajima species as a result of Event 7</td>
<td>$H_{9b}$: Diversification of lineages in Iheyajima and Okinawajima does not coincide with Event 7</td>
<td>Divergence timing between the two lineages coincides with timing of Event 7 at 1.8–0.01 Ma</td>
<td>Iheyajima and Okinawajima species are not sister; and/or divergences pre-date 1.8 Ma</td>
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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 Cretaceaothela 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; Ototuji 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., Ototuji 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’s 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 (Qionghthela) 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
**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 Miocene (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
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 H1a, tmrca of Heptathela + Ryuthela (26.6–18.4 Ma; Fig. 3, Table S3) coincides with EAM island separation from Asia (Ujiıı, 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 H2b, and H4b (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 H5a (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 H5b (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 Ryukyu Heptathela lineages from the northern Ryukyu plus
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 H5a, 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 H5a (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 H8a (Table 1). Finally, Iheyajima species (*H*. sp2, *H*. sp3) are nested within Okinawajima species, and split from *H.*
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 H9a (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 Rynathela (Figs 2 & 3) indicates that Rynathela 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 Ryuku 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 vicariance 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 mygalomorph 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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REFERENCES


**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 Matjaz 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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