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ORIGINAL ARTICLE

Phylogeography of the giant wood spider (*Nephila pilipes*, Araneae) from Asian–Australian regions

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ABSTRACT

Aim There are currently few population genetic studies on widely distributed SE Asian terrestrial organisms. We have studied the genetic diversification pattern of the giant wood spider, *Nephila pilipes* (Araneae: Tetragnathidae) to see whether fluctuations in rain forest extents generated by Quaternary climatic changes left signatures on populations of this agile terrestrial arthropod.

Location The collecting localities were distributed in the following seven regions: (1) N Australia; (2) India (Calcutta, Karziranga and Sukna); (3) SE Asia (N Vietnam, Malaysia, Singapore and Bali); (4) SE China (Fujian, Guandong, Hong Kong and Hainan); (5) SW China (Guangxi and Yunnan); (6) E Asian islands (Ryukyu islands and Taiwan); and (7) the Philippine Islands.

Methods A total of 374 specimens were collected from the East Asian continent and islands, SE Asia, India, and northern Australia. Mitochondrial cytochrome oxidase I gene partial sequences were used as the molecular marker to infer the phylogeographic diversification patterns.

Results From the specimens collected, 67 haplotypes were identified, which could be grouped into five major clades. The dominant clade contained populations in regions ranging from Okinawa to Bali (spanning a distance of more than 4000 km), but their genetic variations were not structured and were not significantly associated with geographical distances. Three clades contained specimens collected from peripheral regions of the distribution range of *N. pilipes*, such as India, N Australia, and NE Asia. Members of the clade distributed in NE Asia were sympatric but those of the clades distributed in Australia and India were allopatric with those of the dominant clade.

Main conclusions The results of this study indicate that, during Quaternary glacial periods, the rain forests in SE Asia might have been more or less continuous and thus generated an unstructured genetic diversification pattern of *N. pilipes* inhabiting this region. However, during such periods, populations in peripheral regions such as India, N Australia and NE Asia might have been isolated in refugia, thus accounting for the observed genetic divergence from populations in the SE Asian region.

Keywords

Asia, Australia, giant wood spider, *Nephila*, phylogeography, population genetic structure, Quaternary climate change, rain forests, SE Asia, Tetragnathidae.

INTRODUCTION

Recently, the effects of Quaternary climatic changes on genetic diversification patterns of organisms have received much attention. However, most relevant studies have been conducted on organisms inhabiting temperate regions (Hewitt, 1996, 1999, 2000; Comes & Kadereit, 1998; Taberlet *et al.*, 1998). Studies on SE Asia, an area exhibiting complex historical geological and climatic changes (Hall, 1998), are rare. During the Quaternary, the climate became drier and more seasonal

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(Bush & Philander, 1999; An, 2000; Kershaw *et al.*, 2001; Bush, 2002; Bush & Fairbanks, 2003), and in many areas of the Sunda shelf the rain forests were replaced by pine forests or savanna (Morley, 2000; Woodruff, 2003). In the glacial periods, the tropical forests retreated to regions near the equator, but in certain interglacial periods the tropical forests might have extended as far north as Korea and Japan (Morley, 2000; Woodruff, 2003). Therefore, during the Quaternary there were dramatic changes in Asia in geological, climatic and vegetation patterns, and such changes would have generated remarkable genetic diversification patterns in terrestrial organisms inhabiting the tropical rain forests of this region.

Currently, there are few phylogeographic studies on organisms inhabiting SE Asia, and most relevant studies conducted in this region have been on widely distributed marine organisms (reviewed by Benzie, 1998). Although some studies have been performed on widely distributed Asian terrestrial organisms, the great majority of them examine interspecific-level phylogeography (e.g. shrews, Ruedi *et al.*, 1998; cockroaches, Maekawa *et al.*, 2001; frogs, Brown & Guttman, 2002; and stone oak, Cannon & Manos, 2003) instead of intraspecific-level genetic divergence patterns. How Quaternary climatic changes might have affected the intraspecific diversification pattern of a terrestrial tropical forest-dwelling organism that is widely distributed in Asia has not hitherto been studied.

In this paper we study the phylogeography of the giant wood spider, *Nephila pilipes* (Fabricius 1793) (Araneae: Tetragnathidae), a large spider widely distributed across Asia and Australia (Murphy & Murphy, 2000). *Nephila pilipes* is a brightly coloured spider, which constructs large orb webs in the understorey of tropical/subtropical forests (Murphy & Murphy, 2000). Several studies had been conducted on its ecology (Tso & Severinghaus, 1998), foraging behaviour (Tso *et al.*, 2002, 2004), silk biology (Tso *et al.*, 2005), and local genetic diversification pattern (Lee *et al.*, 2004). *Nephila pilipes* is distributed in an area spanning E Asia, India, SE Asia, W Pacific islands and N Australia (Platnick, 2006). Since *N. pilipes* is obligate to tropical forests (Murphy & Murphy, 2000), the impacts of Quaternary climatic changes on tropical forests in Asia should have left clear signatures in the genetic diversification patterns of this organism.

In this study we first evaluated whether Quaternary glacial events had generated diversification in *N. pilipes* inhabiting Asian/Australian regions. Currently, the northern distribution limit of *N. pilipes* is southern Japan and the southernmost limit is northern Australia (Platnick, 2006). However, in Quaternary glacial periods the ranges of tropical forests, which are the potential habitats of *N. pilipes*, were much reduced, and peripheral regions in the current distribution range had quite different climates (Bush & Philander, 1999; Bush, 2002; Bush & Fairbanks, 2003) and vegetation (Morley, 2000; Woodruff, 2003). During these time periods, areas such as the E Asian continent and E Asian islands (e.g. Ryukyu Islands and Taiwan) were covered by pine forests or savanna (Zhou *et al.*, 1996; Kimura, 2000) and thus were not suitable for this organism. If there were populations surviving in refugia located in these peripheral regions, then current peripheral populations should exhibit the signatures of such differentiation. Another possibility is that peripheral populations were completely distinct during global cooling owing to the dramatic changes in climate and habitats, and current populations were dispersed from those of SE Asian regions during subsequent interglacial periods. If this was the case, we would expect either a homogenous or an isolated-by-distance pattern between SE Asian and peripheral populations.

In this study we also evaluated whether the Quaternary climatic changes may have generated genetic diversification in SE Asian populations. Currently, what happened to the vast area of tropical rain forests in SE Asia during Quaternary glacial periods is still under debate. Some researchers have proposed that during glacial periods rain forests on the Sunda shelf existed only in a few refugia located in areas in, for example, parts of Borneo, Sumatra, Java and Indochina (Brandon-Jones, 1998, 2001; Gathorne-Hardy *et al.*, 2002). Others have hypothesized that, although the ranges of rain forests were reduced during the glacial periods, there were still considerable areas of rain forest covering parts of the Sunda shelf. These researchers argued that, although the major climatic pattern were dry and seasonal, there were many wide-ranging river networks on the Sunda shelf (Dodson *et al.*, 1995; Voris, 2000), and rain forests might have developed along these drainage networks and formed a continuous mass (Cannon & Manos, 2003; Woodruff, 2003). If tropical rain forests were much reduced and only existed in isolated refugia, the current *N. pilipes* populations in SE Asia would have derived from the rapid expansion of a few founder populations. Following this scenario we would expect current populations to exhibit a structured diversification pattern and characteristics of rapid population expansion. However, if there were still a more or less continuous mass of rain forests in SE Asia during glacial periods, such stable habitats should have allowed gene flow and the accumulation of haplotypes. Following this hypothesis, we would expect current *N. pilipes* populations to be relatively unstructured and to carry no characteristics of a rapid population expansion.

MATERIALS AND METHODS

Specimens examined

Three hundred and seventy-four individuals of *Nephila pilipes* (Fabricius, 1793) were used in this study, including 189 individuals from E Asian islands, the sequences from which were previously analysed by Lee *et al.* (2004). Specimens were widely collected from 47 localities that covered most of the distribution range of *N. pilipes*. The collecting localities (Fig. 1) were distributed in the following seven regions: (1) N Australia (Queensland); (2) India (Calcutta, Karziranga and Sukna); (3) SE Asia (Bali, Malaysia, Singapore and N Vietnam); (4) SE China (Fujian, Guandong, Hong Kong and Hainan); (5) SW China (Guangxi and Yunnan); (6) E Asian islands (Ryukyu islands and Taiwan); and (7) the Philippine Islands. We collected at least five specimens from each colleting site. In each geographic region, specimens were collected from sites separated by at least 600 km (Fig. 1).

DNA extraction, polymerase chain reaction, and sequencing

Specimens were preserved in 95% ethanol, and genomic DNA was extracted from muscles of the cephalothorax or leg using a Puregene DNA isolation kit (Gentra Systems, Inc., Minneapolis, MN, USA). Mitochondrial cytochrome oxidase I (COI) gene partial sequences were amplified using the primer combination LCO-J-1490: 5′-GGT CAA CAA ATC ATA AAG ATA TAT TGG-3′ with HCO-N-2198: 5′-TAA ACT TCA GGG TGA CCA AAA AAA TCA-3′ (Folmer *et al.*, 1994). The reactants were initially denatured for 3 min at 95°C, followed by 30 cycles of 60 s at 95°C, 60 s at 50°C, 60 s at 72°C and then a final extension of 10 min at 72°C. PCR products were assayed by electrophoresis on 1.2% agarose mini gel and were visualized under UV light after ethidium bromide staining. The target DNA fragments were isolated and purified by the Gel/PCR DNA Fragments Extraction Kit (Geneaid, Taiwan). The purified PCR products were sequenced using the BigDye terminator cycle sequencing kit and analysed on an ABI 3100 or 3730 automated DNA sequencer (Applied Biosystems, Foster City, CA, USA).

Sequence analysis and haplotype relationships

Chromatograms and contiguous alignments were edited using Seqman version 4.00 (DNAstar, Madison, WI, USA). All partial sequences of the mitochondrial COI gene were aligned with the ClustalX program (Thompson *et al.*, 1997) using default multiple alignment parameters (because there was no gap in the data set) and then were converted to the fasta format by BioEdit version 4.7.8 (Hall, 1999). Phylogenetic trees were reconstructed by neighbour-joining (NJ) and maximum parsimony (MP) methods implemented in paup* version 4b10 (Swofford, 2001). In addition, the Bayesian method was used to reconstruct the phylogeny. Prior to phylogenetic tree construction, we tested which substitution model best fitted our COI sequence data. We applied the likelihood ratio test for goodness of fit of the nested substitution models using modeltest version 3-06 (Posada & Crandall, 1998). Equal-weighted parsimony analyses were first performed with a heuristic search and trees were obtained by random addition followed by options of tree-bisection-reconnection (TBR) branch swapping and an initial Maxtree setting of 200. Only minimal-length trees were saved, and zero-length branches were collapsed. A 50% majority rule was applied to construct the consensus trees. Bootstrap supports (Felsenstein, 1985) for each clade within the phylogram and consensus tree were estimated using 1000 replications.

The NJ method was conducted using the HKY 85 model (chosen by modeltest) of character substitution with 1000 bootstrap replications. Bayesian analysis was performed with MrBayes 2.01 (Huelsenbeck & Ronquist, 2001) under the model selected by modeltest. We ran four Markov-chain Monte Carlo analyses starting with random trees for each of the four simultaneous chains for 5,000,000 total generations, with a print frequency 1000 and burn-in of 3000 generations. The parameter values from each run were similar, suggesting that the chains were run for a sufficient number of generations to sample adequately the posterior probability landscape. We used species from the subfamily Nephilinae to root the phylogenetic tree. Species from two sister genera of *Nephila*, *Herennia ornatissima* (Doleschall, 1859) and *Nephylengys malabarensis* (Walckenaer, 1841), were used as the outgroups according to the phylogeny based on morphology data in Zhu *et al.* (2003).

Nested-clade phylogenetic analysis

In addition to the phylogenetic relationships of haplotypes, we used nested-clade phylogenetic analysis (NCPA, Templeton, 1998, 2004) to evaluate whether significant associations existed between genetic divergence and geographic distribution and to infer potential causes of these associations. This analysis was performed by means of the following three steps. In the first step we subdivided the known phylogenetic network into component parts and then analysed each one in turn. The network of haplotype relationships was constructed by the TCS procedure (tcs version 1.21, Clement *et al.*, 2000), and the nested rule followed Templeton *et al.* (1987, 1992). In the next step we identified the nested clades showing significant geographic associations. The association patterns between nested clades and geographic information (longitude and latitude) were analysed using the program geodis version 2.0 (Posada *et al.*, 2000). The chi-square values of contingency analyses in each clade, the significance of clade distance (D_C), nested-clade distance (D_N), and the difference between D_C (or D_N) of interior (I) vs. tip (T) clades [namely $(I–T)D_C$ (or $(I–T)D_N$)] were calculated. The significance of these values was determined by permutation tests. In the final step the inference key provided by Templeton (2004) was used to determine the evolutionary mechanisms underlying the observed nested patterns.

Analyses of regional population demographic changes

One of the limitations of NCPA is that it cannot detect certain evolutionary processes that may have occurred within each clade, such as demographic changes (Templeton, 1998). The results of Lee *et al.* (2004) showed that, despite the presence of geographic barriers such as mountains, *N. pilipes* populations spanning an area of 500 km were genetically unstructured. Therefore in this study we regarded specimens collected from sites spanning less than 500 km as members of the same regional population. We used mismatch distribution (Hudson *et al.*, 1992) to evaluate the demographic history of 12 regional

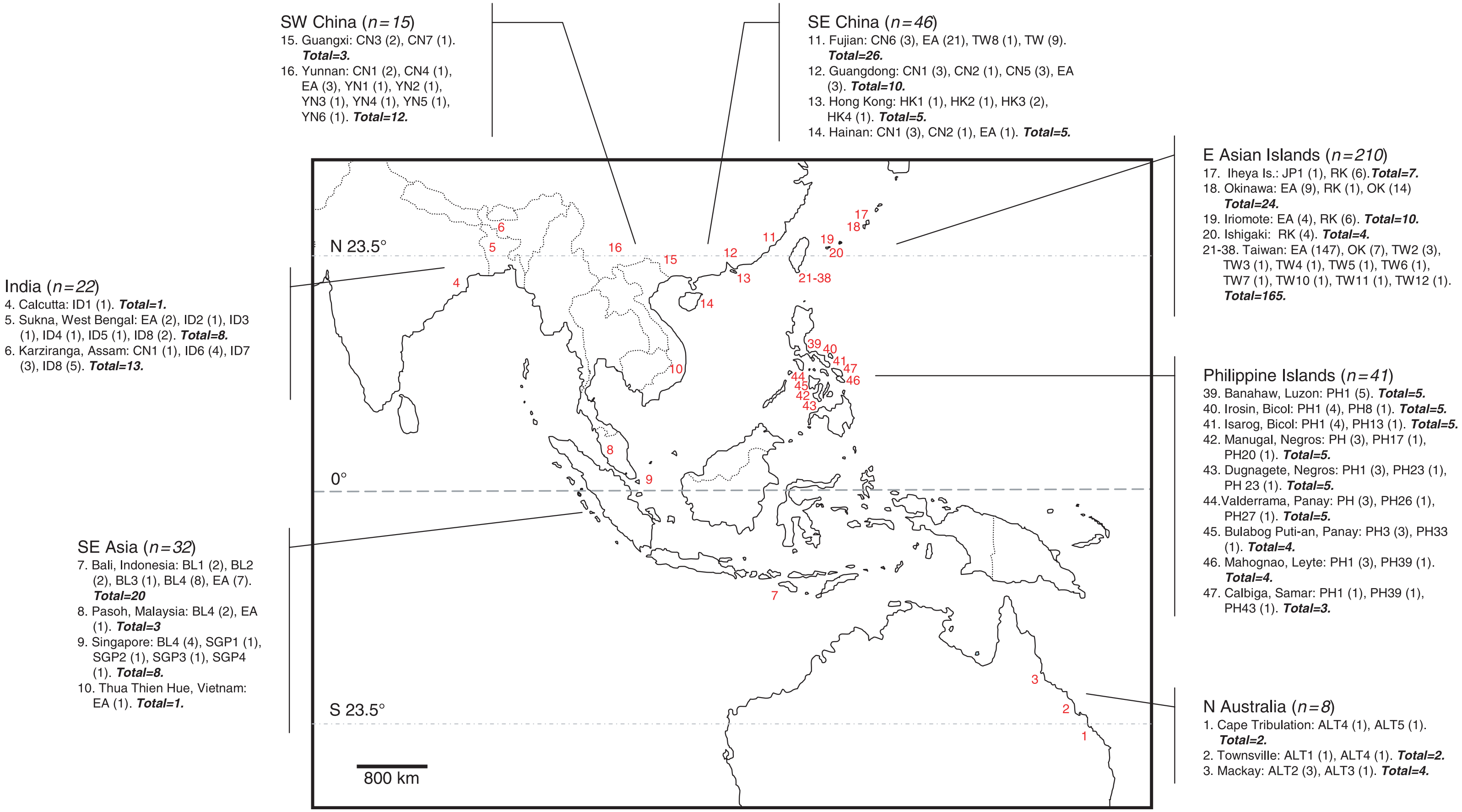


Figure 1 Map of the Asian/Australian regions showing the collection locations of *N. pilipes* ($n = 47$). The number of specimens collected from each major geographic region is shown in italics, and the number in parentheses indicates the sample size of haplotypes. Location codes: (1) N Australia: 1 – Cape Tribulation, Australia, 2 – Townsville, Australia; 3 – Mackay, Australia; (2) India: 4 – Calcutta, 5 – Sukna, West Bengal, 6 – Karziranga, Assam; (3) SE Asia: 7 – Bali, Indonesia, 8 – Pasoh, Malaysia, 9 – Singapore, 10 – Thua Thien Hue, Vietnam; (4) SE China: 11 – Fujian, 12 – Guangdong, 13 – Hong Kong, 14 – Hainan; (5) SW China: 15 – Guangxi, 16 – Yunnan; and (6) E Asian islands: 17 – Iheya Island, Japan; 18 – Okinawa Island, Japan; 19 – Iriomote Island, Japan; 20 – Ishigaki Island, Japan. 21–38 – Taiwan; (7) the Philippine Islands: 39 – Banahaw, Luzon, 40 – Irosin, Bicol, 41– Isarog, Bicol, 42 – Manugal, Negros, 43 – Dugnagete, Negros, 44 – Valderrama, Panay, 45 – Bulabog Puti-an, Panay, 46 – Mahognao, Leyte, 47 – Calbiga, Samar.

populations distributed in India ($n = 20$); SW China (Yunnan, $n = 12$); SE China (Guangdong, $n = 16$, Fujan, $n = 23$ and Hainan, $n = 5$); E Asian islands (Okinawa, $n = 15$, Iriomote and Ishigaki, $n = 15$, Taiwan, $n = 179$); SE Asia (Bali, $n = 20$, Singapore, $n = 10$); Australia ($n = 8$); and the Philippine Islands ($n = 41$) (Fig. 4). Analyses of the frequency distribution of pairwise differences in mtDNA sequences were performed using ARLEQUIN version 2.000 (Schneider *et al.*, 2000). Slatkin & Hudson (1991) showed that, when the population has experienced a recent demographic expansion, the mismatch distribution will have a smooth unimodal distribution. In contrast, the distribution is usually multimodal if populations are more or less stable and have not experienced a recent rapid expansion.

RESULTS

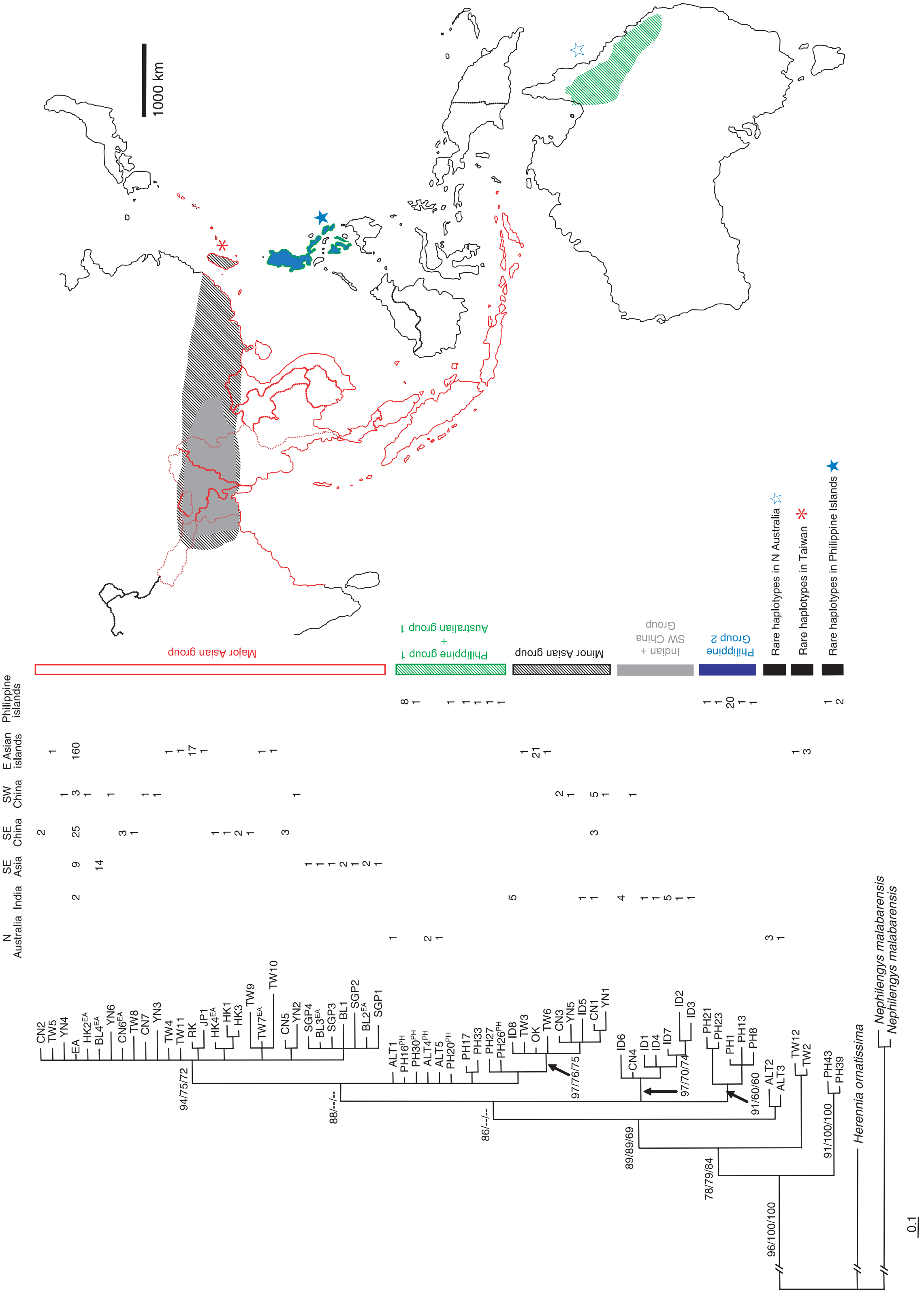
Sequence analyses and haplotype relationships

Partial mtDNA COI gene sequences of 374 *N. pilipes* specimens were used in the analyses. Among the 617 bp obtained, 106 were variable sites, G + C content was 0.306, and there was no indel in the data set. The overall genetic divergence among haplotypes was 1.61 ± 0.96%, and the maximum value was 5.35%. Considering the wide geographic range of *N. pilipes* (from southern Japan in the Northern Hemisphere to northern Australia in the Southern Hemisphere, spanning a distance of 6500 km), the genetic divergence among haplotypes was relatively low. From the sequences of 374 individuals, 67 haplotypes were identified, and we deposited these haplotypes in GenBank (see the Appendix 1 for accession numbers). The haplotype diversity was 0.707 ± 0.026, and the nucleotide diversity was 0.00768 ± 0.00056. The best-fit model chosen by MODELTEST was the TrN + I + G model. We used this model in Bayesian and NJ analyses. The phylogenetic trees obtained by Bayesian, NJ, and MP methods were quite similar, and they exhibited identical haplotype relationships (Fig. 2). In the *N. pilipes* ingroup, in the basal position were three small clades distributed in N Australia (ALT2 and ALT3), Taiwan (TW2 and TW12) and the Philippines (PH39 and PH43). Among the 374 specimens collected from Asian and Australian regions, only 10 individuals carried these haplotypes, and they exhibited no obvious morphological variations. The rest of the specimens collected from vast areas in E Asia, SE Asia, India and N Australia formed a monophyletic clade, and nested in it were five major groups. The first (the major Asian group, Fig. 2) contained 30 haplotypes and 265 individuals, which inhabited most Asian regions, namely India, E Asian islands, SE China, SW China, and SE Asia. Haplotype EA was the most widely distributed haplotype and it inhabited almost all of the geographic areas we sampled in this study except Australia and the Philippine Islands (Fig. 2). Compared with the major Asian group, the other four groups contained far fewer individuals and smaller numbers of haplotypes. The second group (the India + SW China group, Fig. 2) contained 7 haplotypes and 14 individuals. Most of them were distributed in India, but one was from Yunnan in SW China (CN4). In the phylogenetic tree (Fig. 2) the Australian haplotypes and part of the Philippine haplotypes did not cluster as a monophyletic clade. However, in the NCPA network they were all nested within one clade (Fig. 3, clade 3-3). Therefore, in the phylogenetic tree we named this group the Philippine group 1 + Australian group 1. The fourth group (the minor Asian group, Fig. 2) contained 9 haplotypes and 42 individuals, forming a monophyletic clade sister to the major Asian group. Most members of the minor Asian group were distributed in the E Asian islands, the northern distribution boundary of *N. pilipes.* Other members of this group were distributed in the E Asian continent, ranging from E China and SW China to India. The final group is the Philippines group 2, which formed a monophyletic clade sister to other groups.

Nested-clade phylogenetic analysis

Networks were constructed from these 67 haplotypes using the program TCS 1.21, and we estimated the 95% plausible set of cladograms following the rule of Templeton *et al.* (1992). Templeton *et al.* (1992) suggested that, if haplotypes exhibited ambiguous relationships such as forming a loop, they could be pooled as a 0-step clade in the network. In this study we pooled eight haplotypes (BL2, BL3, BL4, HK2, CN6, TW7, TW8, and EA) and named the resulting group EA in clade 3-1. In addition, haplotypes ALT4, PH16, PH20, PH26 and PH30 were also pooled in clade 3-3, and this group was named PH. The nested pattern had three levels, and the clustering patterns of haplotypes in the network were congruent with the topology of the haplotype phylogram (Fig. 2). The rare haplotype clades containing TW2/TW12 and PH39/PH43 formed two isolated basal groups, and the rest of the haplotypes were nested into five major clades (Fig. 3). The first clade, 3-1, was composed of haplotypes of the major Asian group in Fig. 2, members of which inhabited areas ranging from Okinawa, Japan to Bali, Indonesia and India (Fig. 3). Clade 3-2 was composed of haplotypes of the Indian and SW Chinese groups in Fig. 2. Clade 3-3 consisted of haplotypes from the Philippines and N Australia (Philippine group I and Australian group I in Fig. 2). Clade 3-4 contained the rest of the Philippine haplotypes, other than those in clade 3-3. The fifth clade, clade 3-5, was composed of the haplotypes of the minor Asian group in Fig. 2. All of its members inhabited the E Asian continent and islands and were sympatric with those of clade 3-1.

The statistical results of the nested cladistic analyses of geographical associations following the inference key of Templeton (2004, http://darwin.uvigo.es/software/geodis.html) are given in Table 1. For the total cladogram, two possible phylogeographic scenarios were inferred. The overall population differentiation pattern might have been generated either by a long-distance colonization, possibly coupled with subsequent fragmentation, or by past fragmentation followed by range expansion. Among the five 3-step clades, three of them (clades 3-2, 3-3 and 3-5) exhibited a significant


1000 km
N Australia
India
SE Asia
SE China
SW China
E Asian islands
Philippine islands
Major Asian group
Philippine group 1 + Australian group 1
Minor Asian group
Indian + SW China Group
Philippine Group 2
Rare haplotypes in N Australia
Rare haplotypes in Taiwan
Rare haplotypes in Philippine Islands
94/75/72
88/--/--
86/--/--
97/76/75
89/89/69
97/70/74
91/60/60
78/79/84
91/100/100
96/100/100
Herennia ornatissima
Nephilengys malabarensis
Nephilengys malabarensis
0.1

relationship between genetic divergence and geographic distance (Table 1). However, in the widely distributed clade 3-1 and the Philippines clade 3-4 there was no significant geographic association of genetic variances. Among the lower-level clades that exhibited genetic or geographic variations, only in four of them could the null hypothesis be rejected (1-27, 1-30, 2-1 and 2-15, Table 1). The biological implications of the step-3 clades and the clades nested within them were quite different. In clade 3-1, no significant geographic association of genetic divergence was found. However, clade 2-1, nested within 3-1, was inferred to exhibit restricted gene flow but with some long-distance dispersal. Clade 3-2 (distributed in India and SW China) was inferred to have gone through restricted gene flow, with isolation by distance. Clade 3-3, however, cannot be given a conclusive inference because of the insufficient sample size of specimens from areas between the Philippine Islands and Australia. Clade 3-4 members also had no geographic association in their genetic divergences, and they were only distributed in the Philippine Islands. Members of clade 3-5 (the minor Asian group in Fig. 2) were sympatric with those of clade 3-1 and were inferred to have experienced allopatric fragmentation. Several lower-level clades nested in 3-5 were inferred to exhibit various phylogeographic scenarios. According to the inference key, members of clade 2-15 had experienced contiguous range expansion and were distributed in the E Asian continent (Gaungdong and Guangxi, China). Clade 1-27 was inferred to have experienced past fragmentation or long-distance colonization, and its members were distributed in either E Asian islands (Okinawa and Taiwan) or India. Because clade 1-27 was an interior clade nested in 2-14, it could be inferred to be older than the tip clade 2-15.

Analyses of regional population demographic changes

The 12 regional populations collected from seven major geographic areas did not seem to carry signatures of recent population expansions. None of the mismatch frequency distributions of regional populations showed the smooth unimodel curve indicative of recent population expansion, as described in Rogers & Harpending (1992) (Fig. 4). These results indicate that *N. pilipes* populations in the Asian/Australian regions did not go through a recent and rapid population demographic change.

DISCUSSION

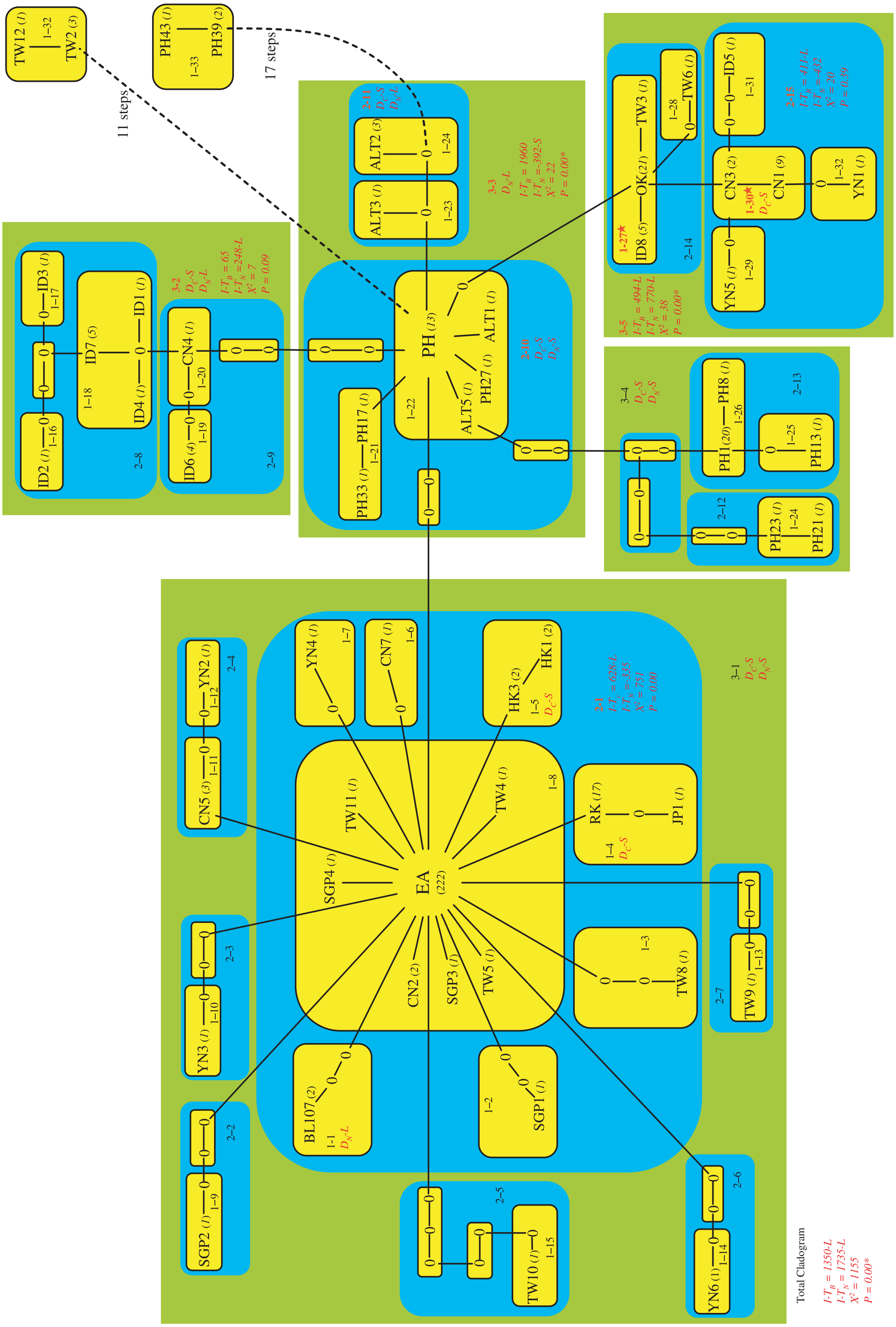
Genetic diversification patterns of *Nephila pilipes*

The overall genetic diversification pattern of *N. pilipes* inhabiting Asian/Australian regions can be depicted as dominated by poorly differentiated and unstructured populations, with patches of diversified populations in peripheral regions of the current distribution of *N. pilipes*. The most dominant clade, clade 3-1 (Fig. 3), contained specimens collected from areas ranging from Okinawa, Japan to Bali, Indonesia, spanning a distance of more than 4000 km. Clade 3-1 exhibited the highest number of haplotypes and thus represented the most dominant group of *N. pilipes* inhabiting E and SE Asian regions. Despite the wide distribution of its members, clade 3-1 was unstructured, with low molecular divergence and exhibiting no sign of rapid expansion in various regional populations (Table 1 and Fig. 4). Most members of the other clades were found in peripheral regions of the distributional range of *N. pilipes*.

While some clade 3-2 haplotypes were distributed at the southern end of the distribution range of *N. pilipes* (N Australia), those of clades 3-3 and 3-5 were found at the north-western (India) and north-eastern (E Asian islands) distributional limits, respectively. Clade 3-3, an interior clade in relation to the other four clades, was inferred as more ancestral than the others. The members in this clade were distributed in two very distant geographic areas, the Philippine Islands and N Australia, and were allopatric with members of clade 3-1. We cannot conclude a solid inference about this clade because there were too few sampling sites between Australia and the Philippine Islands. Clade 3-4 was distributed only in the Philippine Islands, mixed with the Philippine members of clade 3-3. Among the haplotypes of clade 3-4, there was no significant relationship between geographic distances and genetic distances.

In addition to the aforementioned five major clades, a small number of haplotypes inhabiting Australia, Taiwan and the Philippine Islands constituted three clades that were deeply divergent from all the other haplotypes (Fig. 2). This result indicates that in the Asian/Australian regions there were small number of individuals carrying haplotypes that diverged very early from all the others and that were distributed sympatrically with the derived dominant haplotypes. Although these rare clades might provide insights into the evolutionary history

Figure 2 Phylogenetic relationships reconstructed by Bayesian, neighbour-joining, and maximum parsimony methods based on mitochondrial cytochrome oxidase I partial sequences of *N. pilipes*. The Bayesian phylogram is shown in this figure. For each haplotype, the sample size and distribution pattern are specified. The eight haplotypes that are pooled into 'EA' in the NCPA network are specified by the superscript EA. The five haplotypes that are pooled into 'PH' in the NCPA network are specified by the superscript PH. The statistical support for each clade, indicated above the branches, is of the order of the Bayesian posterior probability and the bootstrap values of NJ/MP. The geographic range of each major clade is indicated by colours. The major Asian clade is indicated in red. The Philippine and Australian groups are indicated by the solid blue areas and the green hashed areas. The minor Asian group is designated by the black hashed area. The Indian group is specified by the grey block. Rare haplotypes, found in three localities, are indicated by the symbols ★, ☆ and ∗. The bar under the phylogram equals 0.1 substitutions per site.



★**Clade 1-27**: ID8 D_C-S, D_N-L ;OK D_C-S, D_C-S; $I\text{-}T_c = 317$, $I\text{-}T_N = \text{-}1476\text{-}S$; $X^2 = 54$, $P = 0.00^*$

★**Clade 1-30**: CN3, D_N-S ; CN1, D_C-L, D_C-L; No tip-interior clade; $X^2 = 1$, $P = 0.04^*$

Figure 3 The network used in the nested-clade phylogeographic analysis. This network was composed of five major clades (clades 3-1, 3-2, 3-3, 3-4 and 3-5) and two isolated clades each containing two rare haplotypes (TW2 and TW12; PH39 and PH43). 'EA' in the centre of clade 3-1 indicates the eight (BL2, BL3, BL4, HK2, CN6, TW7, TW8, and EA) and 'PH' in the centre of clade 3-3 indicates the five (ALT4, PH16, PH20, PH26 and PH30) pooled haplotypes. For those clades exhibiting significant associations of geographic distance and genetic divergence, the statistical results are shown in italic. D_C is the clade distance, which represents the mean geographic distance between the individual carrying the specific haplotype and the geographic centre of all individuals carrying that haplotype. D_N is the nested-clade distance, which represents the mean geographic distance of haplotypes (or clades) descending from the same ancestral haplotype (or clade). $(I–T)$ D_C and $(I–T)$ D_N indicate the difference between D_C (or D_N) of the tip clade and that of its interior clade. 'S' or 'L' superscripts represents significantly smaller or larger values of geographic distance determined by permutation tests. The number in parentheses after the name of the haplotype is the number of individuals in that haplotype.

of *N. pilipes*, their small sample size makes it difficult to derive effective biological inferences through analytical methods. Therefore, in this study, the emphasis is on the genetic diversification patterns of the five major clades.

Possible causes of diversified peripheral populations

Results of NCPA showed that the overall diversification pattern of *N. pilipes* inhabiting Asian/Australian regions was generated either by a past fragmentation followed by range expansion, or by long-distance colonization, possibly with subsequent fragmentations (Table 1). However, NCPA results indicated that the populations of the clade containing the highest haplotype diversity (clade 3-1) were unstructured. Moreover, the haplotype phylogeny also showed that this most widely distributed clade was derived rather than ancestral. Therefore, fragmentation should have occurred before the expansion of population ranges. According to the nesting pattern of Fig. 3, clade 3-3 members represented ancestral forms from which the haplotypes of the other clades were derived. Based upon these results, we propose that areas spanning India, the E Asian continent, SE Asia and N Australia were once inhabited by ancestors of clade 3-3 haplotypes. This hypothesis is plausible because, during certain interglacial periods in the Quaternary, the ranges of tropical rain forests in Asia might have reached as far north of Korea (Morley, 2000; Woodruff, 2003). During such time periods, the highly dispersive *N. pilipes* might have been widely distributed in areas spanning India, E/SE Asia and Australia. However, during the subsequent glacial periods, the ranges of tropical forests were greatly reduced. *Nephila pilipes* populations inhabiting peripheral regions of the distributional range might then have become isolated in various refugia, fragmented into several groups, and become isolated in various geographic regions. Some of the peripheral *N. pilipes* populations, such as members of the minor Asian clade, went through a range expansion during the subsequent interglacial periods. Members of the other groups, on the other hand, remained in peripheral regions of the distribution range of *N. pilipes*.

If following this scenario, we still have to explain why only certain clades expanded their ranges, while other clades are still distributed in relatively peripheral regions, such as India, the Philippines and N Australia. Such a pattern might have been generated by the complex palaeoclimatic and palaeogeographic changes in the Asian/Australian regions during the Quaternary. When the glacial periods ended, the tropical forests gradually replaced pine forests or savanna in these peripheral regions. During interglacial periods, members of these clades might have been able to expand their ranges to a certain degree. However, since in the Quaternary there was no land connection between Australia/the Philippines and the SE Asian continent (Morley, 2000), the vast area of sea might have prevented members of Australian and Philippine clades from reaching the Asian continent. Moreover, the dispersal of the members of the Indian clade 3-2 might have been hindered to some degree by the mountains of the Himalaya, and therefore they were partially isolated. Almost all members of clade 3-2 inhabited India, except for one haplotype, which was from SW China. The isolation of Indian populations can be

Table 1 Inferences of clades exhibiting significant associations between genetic divergence and geographic distances in the network (Fig. 3) generated by the key of Templeton (2004)

Clades	Steps	Inferences
Total cladogram	1-2-3-5-6-13-YES-21-NO	Past fragmentation followed by range expansion. Or, long-distance colonization possibly coupled with subsequent fragmentation
3-1		No geographic association of haplotypes
2-1	1-2-3-5-6-7-YES	Restricted gene flow/dispersal but with some long-distance dispersal
3-2	1-2-11-17-4-NO	Restricted gene flow with isolation by distance
3-3	1-19-20-NO	Inadequate geographical sampling
3-4		No geographic association of haplotypes
3-5	1-2-3-5-15-16-NO	Allopatric fragmentation
2-15	1-2-11-YES, range expansion-12-NO	Contiguous range expansion
1-27	1-19-20-2-3-5-15-NO	Past fragmentation or long-distance colonization
1-30	1-19-20-NO	Inadequate geographical sampling

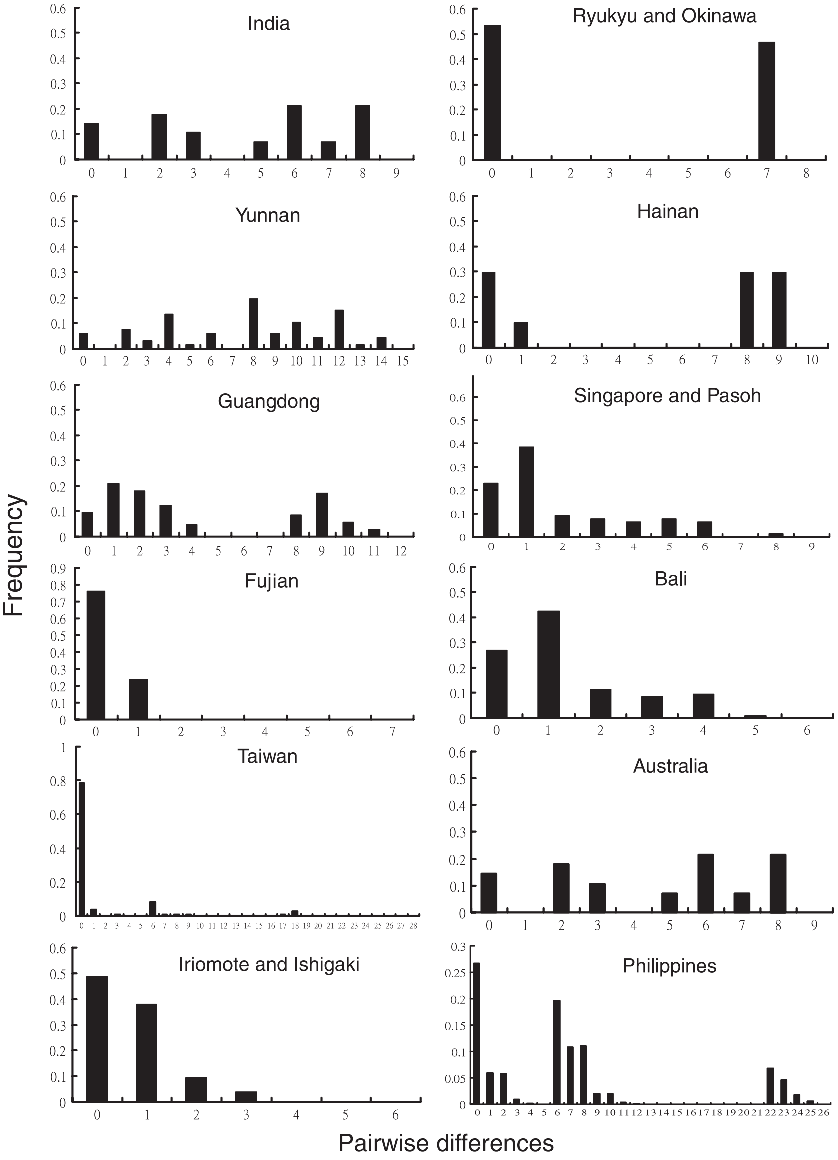


Figure 4 Frequency distributions of pairwise differences between individual haplotypes for 12 *Nephila pilipes* populations.

demonstrated by the significant large difference of D_N value between the tip clade containing the Indian haplotypes (2–8) and the interior clade containing the ancestral SW Chinese haplotype (2–9). This indicates the occurrence of restricted gene flow and isolation by distance between the Indian and other Asian clades.

Members of the minor Asian clade 3-5, on the other hand, had expanded their ranges from E Asian islands into the continent. During the glacial periods in the Quaternary there were land connections between these islands and the E Asian continent (Zhou *et al.*, 1996; Kimura, 2000). Moreover, during interglacial periods, when the sea level rose, there were numerous closely spaced island chains between the E Asian islands and continent (Kimura, 2000). These palaeogeographic events might have facilitated the dispersal of *N. pilipes* from refugia in the E Asian islands into the E Asian continent. Most haplotypes of clade 3-5 are currently distributed in the E Asian continent and islands. Among the members of this clade, the haplotypes in the interior clade 1-27 inhabiting E Asian islands such as Okinawa (the northernmost distribution range of *N. pilipes*) are the most ancestral. According to such nesting patterns we propose that during glacial periods the ancestors of clade 3-3 haplotypes originally isolated in E Asian islands (at that time part of the E Asian continent) evolved into clade 1-27 haplotypes. The nesting pattern in Fig. 3 also shows that haplotypes of the continental clade 2-15 were descendents of the E Asian island clade 1-27. Such a phylogenetic relationship suggests that, after the rise in temperature and recovery of rain forests, members of clade 2-15, the descendents of clade 1-27, dispersed eastwards and southwards into the E Asian continent and eventually reached India. The results of NCPA suggest that the diversification pattern of clade 1-27 might have been generated by past fragmentation and/or long-distance colonization. Since in regions between E Asian islands and India (such as Guandong, Hainan and Yunnan in SW China) there were clade 3-5 haplotypes, a long-distance colonization might be a more plausible explanation for the presence of haplotypes of E Asian origin in India.

Although NCPA is now popularly used in phylogeographic studies, this method has some shortcomings. One major criticism is that the inference part of this method cannot be performed to evaluate alternative scenarios statistically. NCPA can help to determine whether there is a significant association between genetic variation and geographical distance. However, in making inferences of possible mechanisms, there is no way to test alternative hypotheses and to determine the statistical power of the selected scenario (Knowles & Maddison, 2002). Therefore, in addition to interpreting the observed genetic diversification pattern of *N. pilipes* according to the inferences of NCPA, we have to consider whether there are alternative mechanisms. Recently, interspecific molecular introgression has been proposed to be potentially responsible for the observed sequence variation pattern in a number of taxa (Ballard, 2000; Sota *et al.*, 2001; Shaw, 2002). With reference to spiders, such a mechanism has also been reported possibly to be involved in the diversification of the funnel web spider *Tegnaria artica* group (Agelenidae) (Croucher *et al.*, 2004) and eyeless cave spider *Cicurina* (Dictynidae) (Paquin & Hedin, 2004). Therefore, there is a possibility that the diversified *N. pilipes* populations in regions such as India, Australia and S Japan actually reflect signatures of interspecific molecular introgression. There is no way to assess whether ancient molecular introgression occurred in these populations. However, there are several lines of evidence demonstrating that at least contemporary molecular introgression is not responsible for the observed diversification pattern. First, in a molecular phylogeny of the genus *Nephila* constructed by mitochondrial COI and nuclear 28S sequences, *N. pilipes* collected from various regions in Asia and Australia formed a coherent monophyletic group with strong statistical support. This group is in a position basal to all other species of *Nephila* and the molecular distance between it and the other *Nephila* species is great (Tso, unpublished data). Secondly, although *N. pilipes* populations in the Philippines and Australia have similar haplotypes, they are sympatric with different *Nephila* species. Those in the Philippines are sympatric with *N. antipodiana* or *N. laurina*, and those in Australia with *N. edulis* or *N. plumipes* (Platnick, 2006). Since the genetic distances between these species are rather high (estimated from COI and 28S sequences; Tso, unpublished data), it is unlikely that the observed haplotype similarity in *N. pilipes* inhabiting the Philippines and Australia is generated by molecular introgression.

Possible causes of unstructured SE Asian populations

During the lowered sea levels in glacial periods, the major SE Asian islands were connected to the Sunda shelf, and many researchers have debated whether the tropical rain forests were scattered in isolated refugia or formed more or less continuous networks along river banks (Brandon-Jones, 1998, 2001; Gathorne-Hardy *et al.*, 2002; Cannon & Manos, 2003; Woodruff, 2003). In this study, results of NCPA analyses showed that *N. pilipes* populations inhabiting the SE Asian area today are unstructured and that there is no significant association between genetic variation and geographic distance. Moreover, the high number of haplotypes and small DNA divergence among clade 3-1 members indicate that *N. pilipes* populations in the SE Asian region might have been in a more or less stable environment. Therefore, they were unstructured and had accumulated a considerable amount of haplotype diversity. Moreover, results of mismatch distributions further showed that various regional populations in clade 3-1 carried no characteristics of rapid population expansion. All these results are congruent with the hypothesis that there were connected patches of rain forests in SE Asia during glacial periods.

Congruent with such a hypothesis is the genetic diversification pattern of a widely distributed freshwater fish in SE Asia. Dodson *et al.* (1995) examined the effects of Pleistocene sea-level changes in population genetic structures of the freshwater fish *Hemibagrus nemurus*. They found that diversification levels of haplotypes were high, indicating that populations were once isolated. This could have occurred when the sea level rose during interglacial periods, and therefore distant populations were genetically diversified from each other. In addition, the genetic diversification pattern of this species also showed that phylogenetically related groups were distantly separated, while in certain areas highly divergent groups were distributed sympatrically. Dodson *et al.* (1995) argued that, although during interglacial periods the populations were isolated, during glacial periods in the Quaternary there were several wide-ranging major river networks in the Sunda shelf (Voris, 2000). These interconnected water channels might have facilitated the mixing of divergent haplotypes today inhabiting regions separated by a vast area of sea, thus generating the observed genetic diversification pattern in *H. nemurus*. The results of Dodson *et al.* (1995) and this study suggest that during Quaternary glacial periods there were interconnected networks of river channels, which generated the observed genetic diversification patterns of the two SE Asian organisms studied. We may hypothesize that, in the case of the aquatic *H. nemurus*, such interconnected river networks on the Sunda shelf facilitated the mixing of divergent haplotypes between distant populations. In the case of the terrestrial *N. pilipes*, the rain forests along the moist banks of these river networks provided a stable and continuous environment and thus maintained an unstructured diversification pattern among distant populations.

However, since *N. pilipes* and *H. nemurus* were both distributed in SE Asian regions and experienced similar historical events, why do they exhibit such different genetic diversification patterns? While during interglacial periods the rising sea level constituted effective barriers to the freshwater *H. nemurus*, the terrestrial *N. pilipes* seemed to be relatively unaffected by such barriers. A high level of gene flow achieved by aerial dispersal might be responsible for the relatively undifferentiated diversification pattern among SE Asian *N. pilipes* populations. While insects disperse in the adult stage, most spiders disperse in juvenile stages by aerial ballooning (Foelix, 1996). Ballooning behaviours exist among species of 22 families of the Araneomorphae, including the Tetragnathidae (Dean & Sterling, 1985; Decae, 1987; Greenstone *et al.*, 1987). A fully grown *N. pilipes* can reach 5 cm in body length; however, a newly hatched spiderling is as small as a needle tip and can easily achieve aerial dispersal by ballooning. Moreover, during the early stages of development spiderlings usually live on the yolk reserve of the body and do not need to forage for a long period of time (Foelix, 1996). All these traits make *N. pilipes* good dispersers. Ballooning spiders have been reported on ships 300 km from the nearest land and at heights of up to *c.* 5000 m (Gertsch, 1979). In E and S Asia there are prevailing monsoons in both summer and winter. The northeasters dominate during the winter and the southwesterlies prevail during the summer months (Wu, 1998). These monsoons, which run back and forth seasonally across Asia, might have a substantial homogenizing effect on aerial dispersers such *N. pilipes* in this region and might thus have generated the observed genetic diversification pattern.

CONCLUSION

Historical climatic changes and sea-level fluctuations occurred periodically in the SE Asian regions during the Quaternary. The retreat of rain forests in both the Northern and Southern hemispheres during glacial periods might have resulted in the isolation of peripheral *N. pilipes* populations in refugia in India, Australia, the Philippines and NE Asia, thus producing the observed diversification pattern. Although during glacial periods the Asian/Australian regions became drier and more seasonal and were dominated by, for example, pine forests and savannas, there might still have been connected patches of rain forests along the moist banks of river networks on the Sunda shelf. The more or less continuous mass of rain forests, plus the aerial dispersal ability of *N. pilipes*, might have been responsible for the unstructured genetic diversification pattern of populations spanning several thousand kilometres. On the other hand, although our sampling can be regarded as a substantial effort, considering the great distributional range of this dispersive spider, the sample size is still relatively small. In the molecular phylogeny and network there were certain haplotypes with a very small number of individuals but interesting phylogenetic status (such as those in Taiwan, the Philippines and Australia carrying divergent haplotypes). However, their small sample size makes it difficult to provide valid biological inferences. A more comprehensive sampling is needed to identify more rare haplotypes, with greater numbers of individuals, in order to help to determine how these haplotypes were involved in the evolution of *N. pilipes* in the Asian/Australian regions.

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BIOSKETCHES

This work was carried out at **Dr I-Min Tso's** laboratory at the Department of Life Science, Tunghai University, Taiwan. Dr Tso's main research area is the behavioural ecology of spiders, especially visual interactions between orb-weaving spiders in the Tropics/Subtropics and their insect prey. **Mr Yong-Chao Su** is interested in using molecular tools to answer questions regarding evolutionary and speciation mechanisms. **Dr Sin-Che Lee** works on the molecular phylogeny and population genetic structures of marine fishes. **Mr Yung-Hau Chang's** research interest is the taxonomy and systematics of orb-weaving spiders.

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Appendix 1. The accession numbers of the haplotypes used in this study.

Haplotypes		*N*	GenBank accession No.	Haplotypes		*N*	GenBank accession No.	Haplotypes		*N*	GenBank accession No.
1	ALT1	1	DQ779228	26	ID5	1	DQ779246	51	YN4	1	DQ779281
2	ALT2	3	DQ779229	27	ID6	4	DQ779247	52	YN5	1	DQ779282
3	ALT3	1	DQ779230	28	ID7	5	DQ779248	53	YN6	1	DQ779283
4	ALT4	2	DQ779231	29	ID8	5	DQ779249	54	PH1	20	DQ779251
5	ALT5	1	DQ779232	30	JP1	1	DQ779250	55	PH8	1	DQ779264
6	BL1	2	DQ779233	31	RK	17	AY052595*	56	PH13	1	DQ779252
7	BL2	2	DQ779234	32	SGP1	1	DQ779265	57	PH16	8	DQ779253
8	BL3	1	DQ779235	33	SGP2	1	DQ779266	58	PH17	1	DQ779254
9	BL4	14	DQ779236	34	SGP3	1	DQ779267	59	PH20	1	DQ779255
10	CN1	9	AY052588*	35	SGP4	1	DQ779268	60	PH21	1	DQ779256
11	CN2	2	AY052589*	36	OK	21	AY052596*	61	PH23	1	DQ779257
12	CN3	2	AY052590*	37	TW2	3	AY052597*	62	PH26	1	DQ779258
13	CN4	1	AY052591*	38	TW3	1	AY052598*	63	PH27	1	DQ779259
14	CN5	3	AY052592*	39	TW4	1	DQ779272	64	PH30	1	DQ779260
15	CN6	3	AY052593*	40	TW5	1	DQ779273	65	PH33	1	DQ779261
16	CN7	1	DQ779237	41	TW6	1	DQ779274	66	PH39	2	DQ779262
17	EA	199	AY052594*	42	TW7	1	DQ779275	67	PH43	1	DQ779263
18	HK1	1	DQ779238	43	TW8	1	DQ779276				
19	HK2	1	DQ779239	44	TW9	1	DQ779277				
20	HK3	2	DQ779240	45	TW10	1	DQ779269				
21	HK4	1	DQ779241	46	TW11	1	DQ779270				
22	ID1	1	DQ779242	47	TW12	1	DQ779271				
23	ID2	1	DQ779243	48	YN1	1	DQ779278				
24	ID3	1	DQ779244	49	YN2	1	DQ779279				
25	ID4	1	DQ779245	50	YN3	1	DQ779280				

* indicates the haplotypes used in Lee et al 2004