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Source: Zoological Science, 28(1):47-55. 2011.
Published By: Zoological Society of Japan
DOI: 10.2108/zsj.28.47
URL: http://www.bioone.org/doi/full/10.2108/zsj.28.47

ZOOLOGICAL SCIENCE **28**: 47–55 (2011)

Biogeography and Speciation Patterns of the Golden Orb Spider Genus *Nephila* (Araneae: Nephilidae) in Asia

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The molecular phylogeny of the globally distributed golden orb spider genus *Nephila* (Nephilidae) was reconstructed to infer its speciation history, with a focus on SE Asian/W Pacific species. Five Asian, two Australian, four African, and one American species were included in the phylogenetic analyses. Other species in Nephilidae, Araneidae, and Tetragnathidae were included to assess their relationships with the genus *Nephila*, and one species from Uloboridae was used as the outgroup. Phylogenetic trees were reconstructed from one nuclear (18S) and two mitochondrial (COI and 16S) markers. Our molecular phylogeny shows that the widely distributed Asian/Australian species, *N. pilipes*, and an African species, *N. constricta,* form a clade that is sister to all other *Nephila* species. Nested in this *Nephila* clade are one clade with tropical and subtropical/temperate Asian/Australian species, and the other containing African and American species. The estimated divergence times suggest that diversification events within *Nephila* occurred during mid-Miocene to Pliocene (16 Mya-2 Mya), and these time periods were characterized by cyclic global warming/cooling events. According to Dispersal and Vicariance Analysis (DIVA), the ancestral range of the Asian/Australian clade was tropical Asia, and the ancestral range of the genus *Nephila* was either tropical Asia or Africa. We conclude that the speciation of the Asian/Australian *Nephila* species was driven by Neogene global cyclic climate changes. However, further population level studies comparing diversification patterns of sister species are needed to determine the mode of speciation of these species.

Key words: Nephilinae, SE Asia, West Pacific, molecular phylogeny

INTRODUCTION

Members of the genus *Nephila* Leach, 1815 (Araneae, Nephilidae) are globally distributed orb web spiders with a pronounced sexual size dimorphism in which females are conspicuously colorful and considerably larger than males (Fig. 1; Coddington et al., 1997; Kuntner and Coddington, 2009). Females, in some species, use silks containing yellow pigments to build their asymmetric webs (Craig et al., 1996) leading to their common name “golden orb spiders” (Harvey et al., 2007). Many characteristics have made them intensively studied model organisms. Various species have long been used in behavioral ecological studies of foraging, mating, predator-prey interaction, and symbiosis (reviewed by Harvey et al., 2007). They are also intensively used in the studies of sexual selection, such as male-male competition, female choice, sexual size dimorphism, sexual cannibalism, sexual conflict, and sperm competition (e.g., Fromhage and Schneider, 2006; Fromhage et al., 2007; Kuntner et al., 2009a, b; Fig. 1E). Several species of *Nephila* have been intensively studied by molecular biologists to reveal the genetic basis of spider silks (reviewed by Winkler and Kaplan, 2000). Material scientists have also investigated members of this genus for their silk spinning process and silk physical properties (reviewed by Vollrath, 2000). Several research groups have attempted to genetically tailor silks of *Nephila* spiders to mass-produce biomaterial of desired properties (reviewed by Fahnestock et al., 2000).

The genus *Nephila* was initially placed in Araneidae (Levi, 1980). It has been suggested (Levi and Eickstedt, 1989; Coddington, 1990; Coddington and Levi, 1991; Hormiga et al., 1995; Scharf and Coddington, 1997; Griswold et al., 1998) that *Nephila* exhibits apomorphic tetragnathid morphological and behavioral characters and therefore should be treated as a member of Tetragnathidae. Pan et al. (2004) examined the phylogenetic relationships of

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doi:10.2108/zsj.28.47

various araneid genera using the mitochondrial 12S gene, the nuclear 18S gene, and the 3'-end non-repetitive region of the dragline silk MaSp1 gene. The topologies of trees derived from all molecular markers showed that *Nephila* did not group with tetragnathid genera, but was a sister group to araneids. Based on morphological and behavioral evidence, Kuntner (2006) raised subfamily Nephilinae to family level and included genera *Clitaetra*, *Herennia*, *Nephila* and *Nephilengys* in the family Nephilidae. We follow the classification system of Kuntner (2006). Despite the fact that *Nephila* is among the most intensively studied spiders in the world, the only species-level phylogeny is based on morphology and behavior (Kuntner et al., 2008). Studies based on this topology include evolution of size (Kuntner and Coddington, 2009), genital complexity (Kuntner et al., 2009a), and web evolution, (Kuntner et al., 2010). Although some of the intra-nephilid relationships from Kuntner et al. (2008) appear very robust, others, especially within the *Nephila* clade, received low clade support. Thus a more robust tree built on molecular data is desirable.

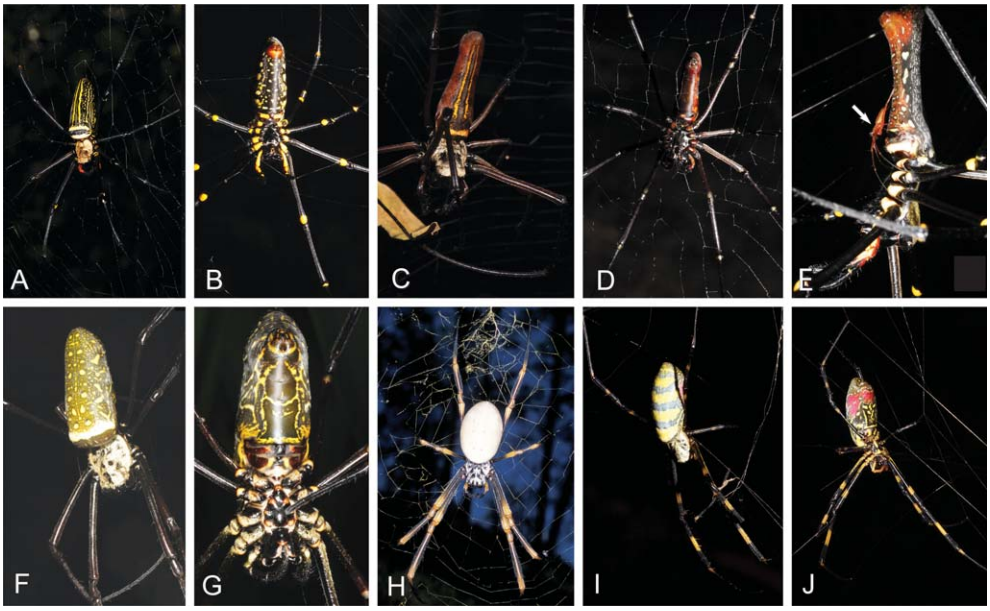

Fig. 1. *Nephila* diversity in Asia and Australia and its extreme sexual size dimorphism: (A–E), *N. pilipes*; **(A–B)**, female of the common color pattern, Taiwan; **(C–D)**, female of the darker color pattern, Taiwan; **(E)**, male (arrow) in copulatory pose on female, Singapore; **(F–G)**, female *N. antipodiana*, Singapore; **(H)**, female *N. plumipes*, Australia; **(I–J)**, female *N. clavata*, Taiwan. Images by M. Kuntner (www.nephilidae.com).

According to their current ranges, the Asian/Australian members of the genus *Nephila* can be categorized as either tropical or subtropical/temperate species (Table 1). In species such as *N. pilipes* and *N. antipodiana*, most populations are widely distributed between the tropics of Cancer and Capricorn, such

Table 1. Distribution, collecting sites, sample sizes, and GenBank accession numbers of spider specimens examined in this study.

Species	Distribution	Collection sites (n)	Accession no.		
			CO1	16S	18S
Nephila antipodiana	China, Philippines to New Guinea, Solomon Is., Queensland; lowlands (N20 - S19)	Labrador Park, Singapore (2)	HQ441924	HQ441949	HQ441969
		Luzon, Philippines (3)	HQ441925	-	-
		Bali, Indonesia (1)	HQ441926	HQ441950	HQ441970
Nephila clavata	India to Japan, low lands in temperate and higlands in subtropics (N33 - N23)	Kaohsiung, Taiwan (2)	HQ441927	HQ441951	HQ441971
		Yunnan, China (2)	HQ441928	-	-
		Saitama, Japan (2)	HQ441929	-	-
Nephila clavipes	USA to Argentina, Sao Tome (N31 - S25)	Gamboa, Panama (1)	HQ441930	HQ441952	HQ441972
		Espirito Santo, Brazil (2)	HQ441930	HQ441952	HQ441972
Nephila constricta	Tropical Africa (N7 - S10)	Mayombe, Congo (1)	HQ441931	HQ441953	HQ441973
Nephila edulis	Australia, New Caledonia (S11 - S38)	Victoria, Australia (2)	HQ441932	HQ441954	HQ441974
		Singleton, Australia (2)	HQ441933	-	HQ441974
Nephila fenestrata	South Africa (S21 - S33)	Eastern Cape, South Africa (2)	HQ441934	HQ441955	HQ441975
Nephila inaurata	South Africa, Mauritius, Rodriguez, Reunion (S21 - S33)	Kwa Zulu-Natal, South Africa (1)	HQ441935	HQ441956	HQ441976
Nephila sp	Hainan, China (N20 - N18)	Hainan, China (1)	HQ441936	-	HQ441977
Nephila pilipes	China, Philippines to Australia, low lands (N26 - S26)	Taiwan (2)	HQ441937	HQ441957	HQ441978
Nephila plumipes	New Guinea, Australia, New Caledonia (S5 - S33)	New South Wales, Australia (2)	HQ441938	HQ441958	HQ441979
		Hornsby, Australia (2)	HQ441939	HQ441959	HQ441979
Nephila senegalensis	West Africa to Ethiopia (N15 - S6)	Mpumalanga, South Africa (1)	HQ441940	HQ441960	-
Herennia multipuncta	India to China, Malaysia, New Guinea	Nantou, Taiwan (1)	HQ441941	HQ441961	HQ441980
Nephilengys malabarensis	India to Philippines, Australia	Nee Soon Swamp Forest, Singapore (4)	HQ441942	HQ441962	HQ441981
Argiope aetheroides	China, Taiwan, Japan	Taitung, Taiwan (1)	HQ441943	HQ441963	HQ441982
Cyrtophora moluccensis	India to Japan, Australia	Iriomote, Japan (1)	HQ441944	HQ441964	HQ441983
Neoscona vigilans	Africa to Philippines, New Guinea	Taiwan (1)	HQ441945	HQ441965	HQ441984
Leucauge magnifica	China, Korea, Taiwan, Japan	Taiwan (1)	HQ441946	HQ441966	HQ441985
Tetragnatha sp	Taiwan	Nantou, Taiwan (1)	HQ441947	HQ441967	HQ441986
Octonoba varians	China, Taiwan, Korea, Japan	Taiwan (1)	HQ441948	HQ441968	HQ441987

as lowland forests of E/SE Asia, W Pacific islands, and N Australia (Yaginuma, 1986; Barrion and Litsinger, 1995; Song et al., 1999; Murphy and Murphy, 2000; Kuntner, 2005, 2008; Harvey et al., 2007). As to the other species, most populations are distributed in subtropical/temperate regions of the tropics of Cancer or Capricorn. For example, *N. clavata* inhabits subtropical and even temperate habitats of E Asia and India (Tikader, 1982; Yaginuma, 1986; Song et al., 1999; Kuntner, 2005, 2008). In the southern hemisphere, *N. plumipes* inhabits New Guinea, Australia, and New Caledonia; and *N. edulis* inhabits mainly Australia (Kuntner, 2005, 2008; Harvey et al., 2007). In addition to the Asian/Australian species, seven species of *Nephila* are distributed in Africa and two in the Americas (Levi and Eickstedt, 1989; Kuntner, 2005; 2008; Platnick, 2010). *Nephila senegalensis* and *N. fenestrata* have broad ranges in the tropical and subtropical Africa, *N. turneri* and *N. constricta* are confined to the African tropics, while *N. inaurata, N. sumptuosa*, and *N. komaci* have narrower ranges in subtropical Africa (Kuntner, 2005, 2008; Kuntner and Coddington, 2009).

We examined molecular phylogeny of selected *Nephila* species emphasizing the Asian/Australian members. Many Asian/Australian *Nephila* species have wide ranges (Song et al., 1999; Harvey et al., 2007; Plantnick, 2010). The most extreme example, *N. pilipes*, is distributed throughout E Asia, SE Asia, S Pacific islands, and N Australia. Recent studies on phylogeography of *N. pilipes* showed a highly homogenous population genetic structure over a vast geographic area coupled with differentiation among populations in subtropical areas and isolated islands (Su et al., 2007). Moreover, local geographic barriers, such as mountains over 3000 m in height and oceans spanning several hundred kilometers, are not effective geographic barriers (Lee et al., 2004). This may be due to the extraordinary dispersal ability of this species, and indeed of *Nephila* in general, during their juvenile stage. Although a fully grown *N. pilipes* can reach 5 cm in body length, a hatched spiderling is smaller than 1 mm and can easily disperse by ballooning (Robinson and Robinson, 1973). Based on these studies, the current species could have speciated near the periphery of the ancestral species' range, applying the diversification scenario inferred from current *Nephila* populations. Speciation in *Nephila* was likely to have been governed by large-scale events, such as global climate changes and isolation of remote landmasses by changes in sea level.

Our first aim was to reconstruct a robust molecular phylogeny of the Asian/Australian *Nephila* species. The phylogeny of Asian/Australian *Nephila* species would establish the basis for future population level studies on their modes of diversification. The ingroup included species widely distributed in tropical Asian/W Pacific areas, and those in subtropical/temperate regions in northern and southern hemispheres, as well as four African and one American *Nephila* species. On the basis of a species phylogeny, our second aim was to assess to what extent the current distribution of Asian/Australian *Nephila* explains the phylogenetic relationships, and to what extent the timing of speciation corresponds to historical climate changes. Because the populations of *Nephila* species in tropical and continental Asia, which is relatively stable climatically, are presumably highly genetically homogenized, we therefore hypothesized that population isolation and the following speciation would have been driven by the historical climate cooling/warming events in subtropical areas. Predictions of this hypothesized speciation scenario are (1) phylogenetically, the subtropical *Nephila* species should be relatively derived; (2) timing of speciation events should correspond to global cooling/warming events; and (3) the reconstructed ancestral distribution of Asian *Nephila* species should be tropical.

MATERIALS AND METHODS

Specimens examined

Eleven *Nephila* species were sampled. We studied three tropical Asian species (*N. pilipes*, an undescribed *Nephila* species from tropical China, and *N. antipodiana*), three subtropical/temperate Australian or Asian species (*N. clavata* from the northern hemisphere, *N. edulis* and *N. plumipes* from Australia), four species from Africa (*N. fenestrata*, *N. inaurata, N. constricta*, and *N. senegalensis*), and one species (*N. clavipes*) from the Americas (Table 1). Recent literature placed either *Nephilengys* or *Herennia* + *Nephilengys* as the sister clade to *Nephila* (Hormiga et al., 1995; Kuntner, 2006; Kuntner et al., 2008); thus one species from each genus were incorporated in the outgroups to *Nephila*. In the outgroup, we also incorporated three species from Araneidae and two species from Tetragnathidae. One species from Uloboridae was used as the ultimate outgroup. For most ingroup species, DNA sequences from at least two individuals were used.

DNA extraction, PCR, 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, Minneapolis, MN, USA). Two regions of the mitochondrial genome, partial sequences of the cytochrome oxidase I (COI) and 16S ribosomal (16S) genes, and partial sequences of the nuclear 18S gene were amplified by polymerase chain reaction (PCR). COI partial sequences were amplified using the primer combination LCO-J-1490: 5'-GGT CAA CAA ATC ATA AAG ATA TAT TGG-3' (Folmer et al., 1994) and C1-N-2735: 5'-AAA ATG TTG AGG GAA AAA ATG TTA-3' (Lunt et al., 1996). Some COI sequences were obtained using the primer combination LCO-J-1490 and HCO-N-2198: 5'-TAA ACT TCA GGG TGA CCA AAA AAA TCA-3' (Folmer et al., 1994), or C1-J-1718: 5'-GGA GGA TTT GGA AAT TGA TTA GTT CC-3' (Simon et al., 1994) and C1-N-2776: 5'-GGA TAA TCA GAA TAT CGT CGA GG-3' (Hedin and Maddison, 2001). 16S partial sequences were amplified using the primer combination LR-J-12887: 5'-CCG GTC TGA ACT CAG ATC ACG T-3' and LR-N-13398: 5'-CGC CTG TTT AAC AAA AAC AT-3' (Simon et al., 1994). The nuclear 18S ribosomal RNA gene was amplified using 18S-ai: 5'-CCTGAGAAACGGCTACCACATC-3' and 18S-b0.5: 5'-GTTTCAGCAACCAT-3' (Tautz et al., 1988). The reactants were initially denatured for 3 min at 95°C, followed by 30 cycles of 60 s at 95°C, annealed for 60 s at either 48°C (16S) or 50°C (COI, 18S), extended for 60 s at 72°C, and then finally extended at 72°C for 10 min. PCR products were assayed with electrophoresis on a 1.2% agarose mini gel and then visualized under UV light after ethidium bromide staining. The target DNA fragments were isolated and purified using a Gel/PCR DNA fragment extraction kit (Geneaid, Taipei, Taiwan). The purified PCR products were sequenced using the BigDye terminator cycle sequencing kit and analyzed on an ABI 3100 or 3730 automatic DNA sequencer (Applied Biosystems, Foster City, CA, USA).

Phylogenetic analyses

The DNA sequences were first checked using the sequence editor, Seqman, *v*.4.00 (DNAstar, Madison, WI, USA), and rechecked against complementary strand sequences. The edited

consensus sequences were exported into a single file. All sequences of 18S, 16S, and COI partial genes were aligned automatically using the Clustal X program *v*.1.83 (Thompson et al., 1997). Multiple alignments were carried out with the default transition weights of 0.5 and gap opening/ gap extension penalties varying over a range including the ratio 8/2, 20/2, 8/4, 24/6, and 24/4. The alignments were optimized manually in Bioedit *v*.7.0.0 (Hall, 1999) and then converted to FASTA format.

Maximum parsimony (MP) analyses were conducted in PAUP*, *v*. 4b10 (Swofford, 2001). Equally-weighted parsimony analyses were first performed with a heuristic search, and trees were obtained by random addition with 1000 replications and with tree-bisection-reconnection (TBR) branch-swapping algorithm. In this analysis gaps were treated as missing data and the initial Maxtree setting was 200. Only minimal-length trees were saved. Bootstrapping was performed 1000 times, then a 50% majority rule was applied to obtain the support of each node. Maximum likelihood (ML) analysis was done in GARLI *v*.0.96 (Zwickl, 2008). MODELTEST *v*. 3.7 (Posada and Crandall, 1998) was used to search the best-fit models for each gene and for the model of the combined data of the genes. Bootstrapping was performed 1000 times in ML. Fifty percent majority rule was applied to obtain the support for each node in MP and ML. Bayesian analysis was performed in MrBayes *v*.3.1 (Huelsenbeck and Ronquist, 2001) under the models selected by MODELTEST. The combined COI, 16S, and 18S sequence data were partitioned and each gene was assigned a best-fit model. Flat prior was used in the Bayesian analysis. The number of generations was first set as 10,000,000 with two Markov chain Monte Carlo (MCMC) chains with a sampling frequency of every 100 generations. The analyses were terminated if the standard error difference between two chains dropped below 0.0001. By plotting log likelihood values in Tracer *v*.1.41 (Rambaut and Drummond, 2008), the appropriate number of burn-in generations was found. The posterior probabilities of each node were saved.

Intraspecific divergence

While many *Nephila* species are extremely widely distributed, genetic divergence of specimens from widely separated localities is lacking for most. The exceptions are the Asian *N. pilipes* (see Lee et al., 2004; Su et al., 2007) and the African/Indian Ocean *N. inaurata* (Kuntner and Agnarsson, unpublished). Therefore, the same COI partial sequence as used in phylogenetic analyses was used to estimate the genetic divergence in *N. antipodiana* and *N. clavata*. The samples in *N. edulis* and *N. plumipes* were not used because the distances between different collecting sites are less than 1000 km. In this part of study localities that had only one specimen were excluded from the analyses (such as *N. antipodiana* from Bali, Indonesia). For *N. antiopodiana*, three samples from Banahaw, Luzon Island, Philippines, and two samples from Singapore were used. The distance between these two sites is ~2300 km. For *N. clavata*, two specimens from Yunnan, China, two from Saitama, Japan, and four from Taiwan were used. The average distance between these collecting sites is ~2430 km. The genetic distances between localities and within localities were estimated with *p*-distance in MEGA *v*.4.0 (Tamura et al., 2007)

Divergence time estimation

Divergence times were estimated by r8s *v*.1.71 (Sanderson, 2004) using ML tree. We used Penalized Likelihood (PL) approach with cross-validation method to date the divergence time of *Nephila* species. Five extinct *Nephila* species are known from Dominican amber from the Miocene (about 20 Mya) (Wunderlich, 2004). Therefore, the origin of *Nephila* can be estimated to be at least 20 Mya. We used this lower bound estimation as the calibration time for the origin of the genus. The smoothing parameter was calculated with cross-validation number of 100 and an increment of 0.1. The lowest chi-square error value indicated the best smoothing log10 value. The output tree with divergence time was saved and visualized in FigTree *v*.1.2 (Rambaut, 2008).

Dispersal and Vicariance Analysis (DIVA)

DIVA analysis was based on our phylogenetic tree reconstructed from combined data. The tropical Asian species *Nephilengys malabarensis* and *Herennia multipuncta* were included in the analysis. A total of six areas were assigned (A: tropical America, B: tropical Africa, C: tropical Asia, D: Northern subtropical Asia, and E: Subtropical Australia). To reconstruct the ancestral distribution pattern within *Nephila*, event-based dispersal-vicariance analysis (DIVA) was conducted (Ronquist, 1996, 1997). DIVA allows the reconstruction of ancestral areas by assigning cost matrix of historical events of vicaricance (cost = 0), sympatric speciation (cost = 0), dispersal (cost > 0), and extinction (cost > 0), without a presumption of area cladograms. This analysis is thus suitable for our data, since a general hypothesis of area relationships on a global scale for species-level phylogeny is usually lacking. The bifurcate maximum parsimony tree constructed by nuclear and mitochondrial gene sequences was specified to generate the input file. Species were treated as the entities of the analysis only if the populations within the species were monophyletic. The reconstruction of ancestral distribution pattern was implemented using the default settings. The reconstructed ancestral areas were then mapped on the molecular phylogenetic tree.

RESULTS

Species phylogeny

Sequences with a combined total length of 2179 bp (1110 for COI, 520 for 16S and 549 for 18S) were used in the phylogenetic analyses. Equally-weighted parsimony analysis showed that among the 976 variable sites, 796 were parsimony informative. Only one most parsimonious tree with a length of 2795 steps was obtained (CI = 0.503 and RI = 0.5838). MODELTEST indicated that the best substitution model for COI and 16S was GTR + G + I, for 18S was GTR + G, and for the combined data matrix was GTR + G + I. These models were used for ML and Bayesian analyses. In the ML analysis, the score of the best tree found was –lnL = –6113.34. The nucleotide relative rates were 0.907 for AC, 5.344 for AG, 2.975 for AT, 2.098 for CG, 4.095 for CT, and 1.000 for GT. Equilibrium state frequencies were 0.291 for A, 0.146 for C, 0.204 for G and 0.359 for T. The estimated alpha parameter was 0.731, invariant site proportion was 0.348. In the Bayesian analysis, the data were partitioned into three genes using the best-fit models for them. In total, 1,000,000 generations were run with a sampling frequency of 100 generations for each chain. Burn-in was set to 5000 after been traced in Tracer *v*.1.41. In total, 20,002 trees were obtained from each chain and 19,002 trees were used to get posterior probabilities for every node.

The topologies of the consensus trees using *Octonoba varians* (Uloboridae) as the outgroup generated by MP, ML, and Bayesian methods were similar, but differed in the statistical supports of the nodes (Fig. 2). Nephilidae was monophyletic with high statistical support in each analysis (support values, MP/ML/Bayesian = 100/100/100). Nephilidae was sister to Araneidae with high support (MP/ML/Bayesian = 98/91/100). All species currently classified as *Nephila* formed a clade with good support (MP/ML/Bayesian = 62/92/100). However, within *Nephila*, MP and ML methods failed to resolve the species relationships within the African +

American clade. In outgroups, the relationship between *Herennia* and *Nephilengys* was poorly supported.

Within *Nephila*, *N. pilipes* and *N. constricta* form a clade sister to all the remaining species, which fall into two geographically distinct clades, one containing the African/American species, and one containing Asian/Australian species (Fig. 2). In the Asian/Australian clade, the northern hemisphere subtropical species *N. clavata* and the northern hemisphere tropical species *N.* sp were not monophyletic. *Nephila* sp was sister to *N. edulis*, a southern hemisphere subtropical species (MP/ML/Bayesian = 100/100/100). The northern hemisphere subtropical *N. clavata* was sister to the tropical, equatorially distributed species *N. antipodiana* (MP/ML/Bayesian = 90/91/90). The southern hemisphere subtropical *N. plumipes* was sister to other Asian/Australian species, but this relationship was poorly supported.

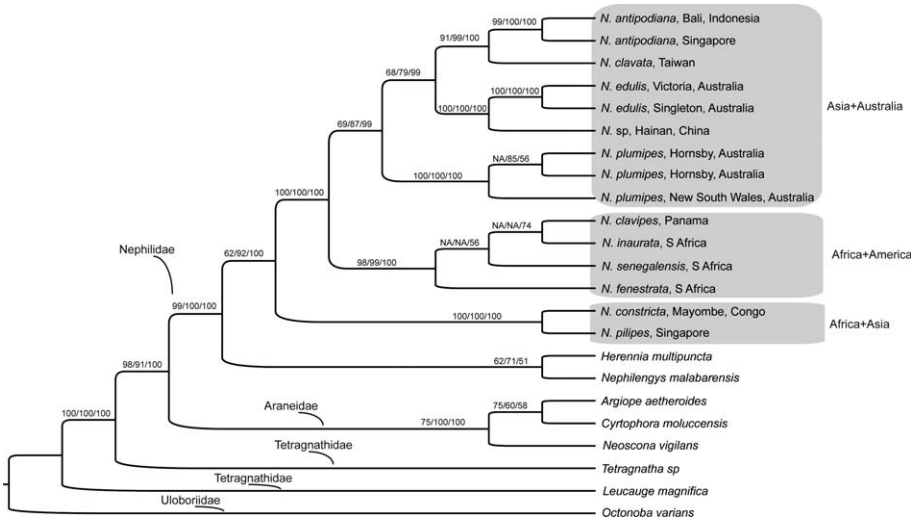

Fig. 2. The phylogeny of the genus *Nephila.* The bootstrap supports of MP and ML (50% majority rule applied) and the posterior probability of Bayesian analysis are shown on each node. The tree shown is the Bayesian tree.

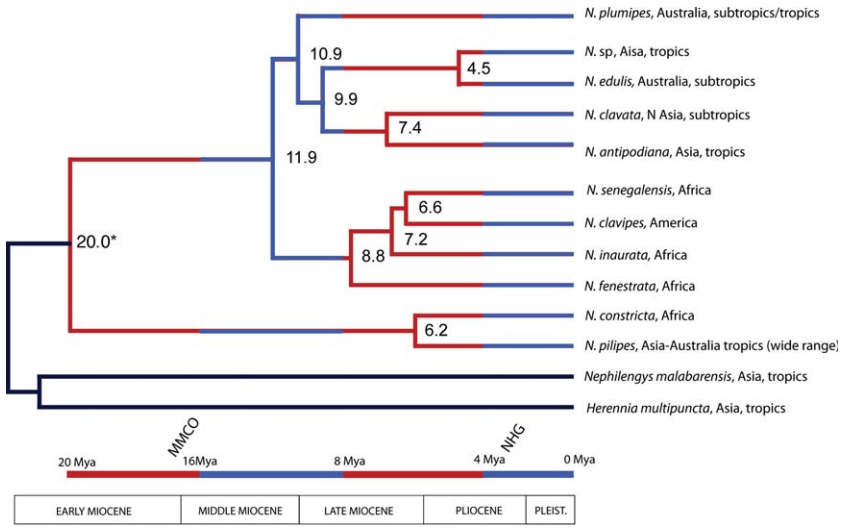

Fig. 3. The estimated divergence time of species of the genus *Nephila.* The red and blue bars indicate the warming (red) and cooling (blue) episodes of the paleoclimate. * indicates the calibration node assigned as 20 Mya. The epoches were defined as Miocene: 23 Mya to 5.6 Mya; Pliocene: 5.6 Mya to 2 Mya; Pleistocene: ~2 Mya, following Zachos et al. (2001). The defined warming and cooling episodes follow Tsuchi (2002), and are similar to the epoches defined in Zachos et al. (2001). MMCO = mid-Miocene Climate Optimum; NHG = N Hemisphere Glaciation.

Intraspecific divergence

COI genetic divergence between *N. antipodiana* from Singapore and Luzon, Philippines was 1.52%, while the average genetic divergence within collecting sites was 0.04%. A relatively small COI genetic divergence was also found in *N. clavata*. Genetic divergence among three localities ranged from 0.00% to 0.87%, with an average of 0.62%. The average within-locality divergence was 0.18%. The samples from Yunnan, China and Saitama, Japan had identical haplotypes.

Divergence time

According to the estimated time by Penalized Likelihood (PL) approach with cross-validation method (smoothing parameter = 0.9), the divergence between the *N. pilipes* + *N. constricta* clade and the other *Nephila* species was dated 11.9 Mya. The subsequent diversification within the Asian/Australian clade was 10.9 Mya for the separation of *N. plumipes* from other Asian/Australian species. The differentiation between the *N. clavata* + *N. antipodiana* clade (northern subtropical + tropical Asian species) and the *N. edulis* + *N.* sp clade (southern subtropical + tropical Asian species) was dated 9.9 Mya (Fig. 3).

Dispersal and Vicariance Analysis (DIVA)

The DIVA results (Fig. 4) showed that at least seven dispersal events were required to generate the current distribution pattern. The ancestral area of Asian/Australian species estimated by DIVA was tropical Asia. In the clade containing African and American species, the American *N. clavipes* was at the derived position and the ancestral area of this clade was designated as Africa. The overall ancestral area of all *Nephila* species examined, excluding the basal *N. pilipes* + *N. constricta*, was tropical Asia + Africa. The ultimate ancestral area of this genus determined by DIVA was tropical Asia or Africa.

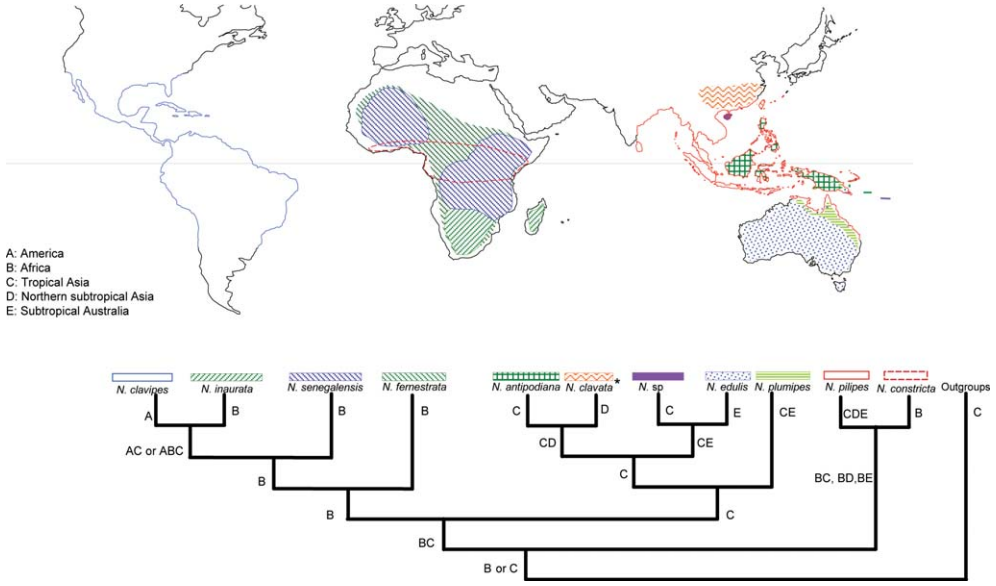

Fig. 4. Geographical distribution and the phylogenetic relationships of the species in genus *Nephila*. The various shades indicate the distribution range of each species. The topology of the phylogenetic tree under the map is inferred from mitochondrial and nuclear evidence. *N. pilipes* is the most widely distributed species. The letters A to E indicate the area codes and DIVA analysis results. *: *N. clavata* is also distributed in India, Japan and mid-elevation areas in Taiwan.

DISCUSSION

Compared with the known phylogeny of *Nephila*, results of our molecular phylogenetic analyses agree in three particulars and disagree in two particulars. 1) They confirm that Nephilidae is sister to Araneidae. Such results are congruent with those reported by Pan et al. (2004), Kuntner (2005, 2006, 2007, 2008) and Kuntner et al. (2008), who concluded that nephilids are not members of the clade previously defined as Tetragnathidae (see also Alvarez-Padilla et al., 2009; *contra* Hormiga et al., 1995). 2) They further confirm the monophyly of the nephilids, which include representatives of *Nephila*, *Nephilengys*, and *Herennia* (Kuntner, 2006; Kuntner et al., 2008). 3) The data support *Nephila* monophyly (Kuntner et al., 2008). 4) They suggest that *N. pilipes* + *N. constricta* is a clade sister to all other *Nephila* species (*contra* Kuntner et al., 2008, who recovered that clade, but had *N. fenestrata* as sister to all other *Nephila*). 5) And they suggest that, excluding *N. pilipes* and *N. constricta*, the *Nephila* species form two geographically-consistent clades, one containing the African/American species and the other the Asian/Australian species (*contra* Kuntner et al., 2008).

In two *Nephila* species, specimens collected from distant localities (spanning > 2000 km) exhibited relatively small intraspecific genetic divergence. In *N. clavata*, specimens from Taiwan, southern China, and Japan exhibited less than 1% divergence in COI sequences. In *N. antipodiana*, specimens from Philippines and Singapore had only 1.52% divergence. The phenomenon of low genetic divergence among conspecific individuals over wide geographic area has been repeatedly found in other *Nephila* species, e.g., *N. pilipes* (using COI gene; Su et al., 2007), *N. edulis* and *N. plumipes* (using allozyme data; Harvey et al., 2007), and *N. inaurata* (Kuntner and Agnarsson, unpublished). We predict that after a careful revision of sufficient specimens using both morphological and molecular characters, the individual specimens with subtle morphological differences distributed in different areas may be determined to belong to the same species, and the number of valid *Nephila* species and subspecies might be substantially reduced.

Estimated divergence time in *Nephila* species

According to the oldest fossil record of *Nephila* (20 Mya, Wunderlich, 2004), it is unlikely that *Nephila* had originated in either Africa or India then entered Asia after the collision of the Indian plate with the Asian continent, which occurred much earlier (45 to 55 Mya; Patriat and Achache, 1984; Dewey et al., 1989). In the Neogene, the relative positions of the major tectonic plates were nearly identical to their present status (Smith et al., 1994), and tropical regions of different continents were separated by either the Pacific or Atlantic Oceans. Therefore, the global distribution of *Nephila* was influenced more by intercontinental dispersal than vicariance.

The separation of the African/American clade and the Asian/Australian clade, (excluding *N. pilipes* and *N. constricta*) was dated around 11.9 Mya, at which time was a severe global cooling event during mid to late Miocene (16 Mya to 8 Mya) after the mid-Miocene Climate Optimum (Zachos et al., 2001). The divergence of *Nephila* species within African/American regions and Asian/Australian regions might have occurred during late Miocene (8 Mya to 6 Mya) to Pliocene (~6 Mya to 2 Mya). At that time there were cyclic global warming/cooling events and periodic fluctuations in ranges of tropical forests on all continents (Morley, 2000; Zachos et al., 2001; Tsuchi, 2002). During interglacial periods, tropical forests could extend as far north as central Japan and Korea (Morley, 2000; Woodruff, 2003). Although there was no permanent land connection between SE Asia and Australia in the Neogene, island chains in between might have facilitated the exchanges of terrestrial biotas (Hall, 1998). During the next global warming/cooling event, i.e., the cooling period in the late Miocene and early Pliocene (8–6 Mya) to the North Hemisphere Glaciation (~4 Mya, Zachos et al., 2001), tropical forests retreated to regions near the equator (Morley, 2000). Changes of tropical forest ranges in both northern and southern hemispheres might be one major factor driving the speciation of Asian/Australian *Nephila* species. To confirm whether such events were responsible for speciation of *Nephila* spiders and other organisms in this region, further studies quantify-

ing and comparing the speciation rates between geological periods with or without cyclic climatic changes should be carried out.

Phylogeny and ancestral areas of Asian/Australian *Nephila*

Our phylogeny differs from the morphology-based results of Kuntner et al. (2008) in recognizing two sister clades, *N. pilipes* + *N. constricta* and the remaining *Nephila* species. Another difference is that our analyses recognized two geographically consistent clades in the latter group, one containing the African/American species and the other the Asian/Australian species. The topology of this phylogeny is congruent with our prediction that the subtropical/ temperate species are in relatively derived positions (Fig. 2). In addition, we identified two pairs of sister species, *N. clavata* + *N. antipodiana* and *N. edulis* + *N.* sp, in the Asian/Australian clade. This result shows that geographic proximity does not necessarily correspond to phylogenetic proximity, and the two tropical species both have a subtropical sister species. Although the range of *N. antipodiana* overlaps with that of *N.* sp, they are not phylogenetically close. Instead, the tropical Asian species *N. antipodiana* is closely related to the northern subtropical/temperate Asian species *N. clavata.* The reconstruction of ancestral ranges (DIVA analysis) indicates that the ancestral range of Asian/Australian clade is in tropical Asia. Therefore, the subtropical/temperate species, *N. clavata* and *N. edulis*, which are not sister species, independently speciated in the areas peripheral to the Asian/Australian tropical regions. The speciation modes of these two pairs of sister species should be further tested by population genetic studies examining their phylogeographic structures.

Potential speciation scenarios of Asian/Australian *Nephila*

Results of our phylogenetic analyses using molecular data emphasizing Asian/Australian *Nephila* species are congruent with the predictions that 1) the subtropical/temperate species are phylogenetically derived; 2) their speciation events correspond to Neogene warming/cooling cycles; and 3) the ancestral range of these species was tropical. Our analyses show that the speciation events did not occur in tropical areas. Instead, the diversification forces in the peripheral areas of distribution ranges of ancestral *Nephila* species might be responsible for the speciation of *Nephila*. However, to evaluate the possible speciation scenarios, one would need to examine the biology and population genetic structure of each tropical/subtropical sister species pair. In *N. pilipes*, this species' genetic diversification pattern can be explained by the peripatric speciation mode (Mayr, 1982). Nevertheless, natural selection might also be involved in other species. For example, the latitudinal and altitudinal distribution patterns of *N. clavata* (Lee, 1964) reflect their adaptations to a colder environment compared with its sister species *N. antipodiana*. We suggest that one lineage of the isolated peripheral ancestral *Nephila* populations might have evolved cold/temperate-adapted traits, diverged from the ancestral tropical populations and eventually speciated into distinct species such as *N. clavata.* On the other hand, Asian/Australian tropical *Nephila*, such as *N. antipodiana* and *N*. sp, might represent other lineages of isolated peripheral ancestral *Nephila* populations that retained the tropic-adapted traits and went through backward invasion process to the tropical areas. Alternatively, it is possible that speciation of these *Nephila* species occurred in tropics and certain lineages shifted their ranges into subtropics. However, widely-distributed *Nephila* species in tropical Asia either exhibited homogenous population genetic structure (*N. pilipes*, Su et al., 2007) or low genetic divergence (*N. antipodiana*, this study). Such phenomena suggest existence of substantial gene flows among various *Nephila* populations in tropical Asia and therefore the probabilities of diversification events should be low. We suggest that the hypothesized cold adaptation of subtropical/temperate *Nephila* species be tested by ecophysiological studies. Furthermore, phylogeographic studies should be carried out on these species pairs to see whether their population genetic structures are congruent with our hypothesized speciation scenarios.

ACKNOWLEDGMENTS

We thank W. H. Chou, J. Chen, A. S. Dippenaar-Schoeman, M. E. Herberstein, D. Li, H. Ono, X. J. Peng, D. de Bakker, and H. Smith for providing *Nephila* specimens. We appreciate the helpful comments from Dr. Daphne G. Fautin. Special thanks are given to all the members of the Behavioral Ecology Laboratory, Tunghai University, Taichung, Taiwan for assistance in logistics, field collection, molecular techniques, and genetic data analysis. This study was supported by grants from the Institute of Zoology, Academia Sinica, Taiwan and grants from National Science Council, Taiwan (NSC 97-2311-B-029 -002 -MY3, NSC 96-2628-B-029 -001 -MY3) to I. M. Tso, and in part by the Slovenian Research Agency and the EU Marie Curie grants to M. Kuntner.

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(Received February 15, 2010 / Accepted June 28, 2010)