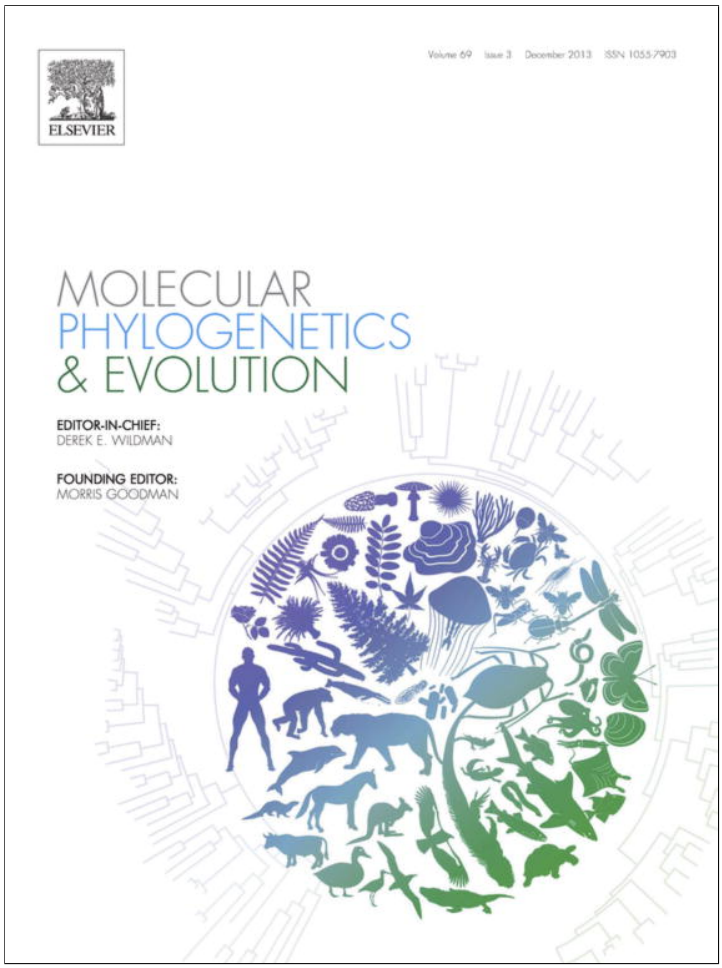

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A molecular phylogeny of nephilid spiders: Evolutionary history of a model lineage

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ABSTRACT

The pantropical orb web spider family Nephilidae is known for the most extreme sexual size dimorphism among terrestrial animals. Numerous studies have made Nephilidae, particularly *Nephila*, a model lineage in evolutionary research. However, a poorly understood phylogeny of this lineage, relying only on morphology, has prevented thorough evolutionary syntheses of nephilid biology. We here use three nuclear and five mitochondrial genes for 28 out of 40 nephilid species to provide a more robust nephilid phylogeny and infer clade ages in a fossil-calibrated Bayesian framework. We complement the molecular analyses with total evidence analysis including morphology. All analyses find strong support for nephilid monophyly and exclusivity and the monophyly of the genera *Herennia* and *Clitaetra*. The inferred phylogenetic structure within Nephilidae is novel and conflicts with morphological phylogeny and traditional taxonomy. *Nephilengys* species fall into two clades, one with Australasian species (true *Nephilengys*) as sister to *Herennia*, and another with Afrotropical species (*Nephilingis* Kuntner **new genus**) as sister to a clade containing *Clitaetra* plus most currently described *Nephila*. Surprisingly, *Nephila* is also diphyletic, with true *Nephila* containing *N. pilipes* + *N. constricta*, and the second clade with all other species sister to *Clitaetra*; this "*Nephila*" clade is further split into an Australasian clade that also contains the South American *N. sexpunctata* and the Eurasian *N. clavata*, and an African clade that also contains the Panamerican *N. clavipes*. An approximately unbiased test constraining the monophyly of *Nephilengys, Nephila*, and Nephilinae (*Nephila*, *Nephilengys*, *Herennia*), respectively, rejected *Nephilengys* monophyly, but not that of *Nephila* and Nephilinae. Further data are therefore necessary to robustly test these two new, but inconclusive findings, and also to further test the precise placement of Nephilidae within the Araneoidea. For divergence date estimation we set the minimum bound for the stems of Nephilidae at 40 Ma and of *Nephila* at 16 Ma to accommodate *Palaeonephila* from Baltic amber and Dominican *Nephila* species, respectively. We also calibrated and dated the phylogeny under three different interpretations of the enigmatic 165 Ma fossil *Nephila jurassica*, which we suspected based on morphology to be misplaced. We found that by treating *N. jurassica* as stem *Nephila* or nephilid the inferred clade ages were vastly older, and the mitochondrial substitution rates much slower than expected from other empirical spider data. This suggests that *N. jurassica* is not a *Nephila* nor a nephilid, but possibly a stem orbicularian. The estimated nephilid ancestral age (40–60 Ma) rejects a Gondwanan origin of the family as most of the southern continents were already split at that time. The origin of the family is equally likely to be African, Asian, or Australasian, with a global biogeographic history dominated by dispersal events. A reinterpretation of web architecture evolution suggests that a partially arboricolous, asymmetric orb web with a retreat, as exemplified by both groups of "*Nephilengys*", is plesiomorphic in Nephilidae, that this architecture was modified into specialized arboricolous webs in *Herennia* and independently in *Clitaetra*, and that the web became aerial, gigantic, and golden independently in both "*Nephila*" groups. The new topology questions previously hypothesized gradual evolution of female size from small to large, and rather suggests a more mosaic evolutionary pattern with independent female size increases from medium to giant in both "*Nephila*" clades, and two reversals back to medium and small; combined with male size

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evolution, this pattern will help detect gross evolutionary events leading to extreme sexual size dimorphism, and its morphological and behavioral correlates.

1. Introduction

The golden orb weaver genus *Nephila* is one of the few groups of spiders that have a substantial appeal outside of the small specialist arachnological community. It is known for the most extreme cases of sexual size dimorphism among terrestrial animals (Fig. 1c)—females are on average 125 times, and up to 500 times, heavier than the small males (Kuntner et al., 2012b)—and this represents a unique case of evolutionary female gigantism because the females have increased in size whereas the males have not (Coddington et al., 1997; Hormiga et al., 2000; Kuntner and Coddington, 2009). Species of *Nephila* also produce gigantic aerial orb webs (Fig. 6a and c) sometimes reaching a meter and a half in diameter (Kuntner et al., 2010b), and these are characteristically composed of shiny golden silk (Craig et al., 1996) and are made of densely spun fibers capable of subduing even vertebrate prey (Sakai, 2007; Nyffeler and Knörnschild, 2013). The sheer size of these webs attracts various kleptoparasitic spiders sometimes vastly outnumbering the host (Agnarsson, 2003, 2011; Higgins and Buskirk, 1998). *Nephila* is pantropical, and some species have ranges that are uncharacteristically extensive for invertebrates, e.g. *N. pilipes* (Fabricius, 1793) occupies mainland forests of south and southeast Asia and Australia, as well as islands as far east as Japan, the Solomons, and Vanuatu (Harvey et al., 2007; Su et al., 2007), while others are restricted to smaller parts of continents, e.g. *N. sexpunctata* Giebel, 1867 to a part of South America and *N. komaci* Kuntner and Coddington, 2009 to small pockets of southeastern Africa (Kuntner and Coddington, 2009). These distributions raise interesting biogeographical and speciation hypotheses relating to the organisms' dispersal abilities (Agnarsson and Kuntner, 2012; Kuntner and Agnarsson, 2011a,b; Lee et al., 2004; Su et al., 2007, 2011), and may suggest that some species are habitat specialists at risk of extinction while others thrive in anthropogenically altered habitats.

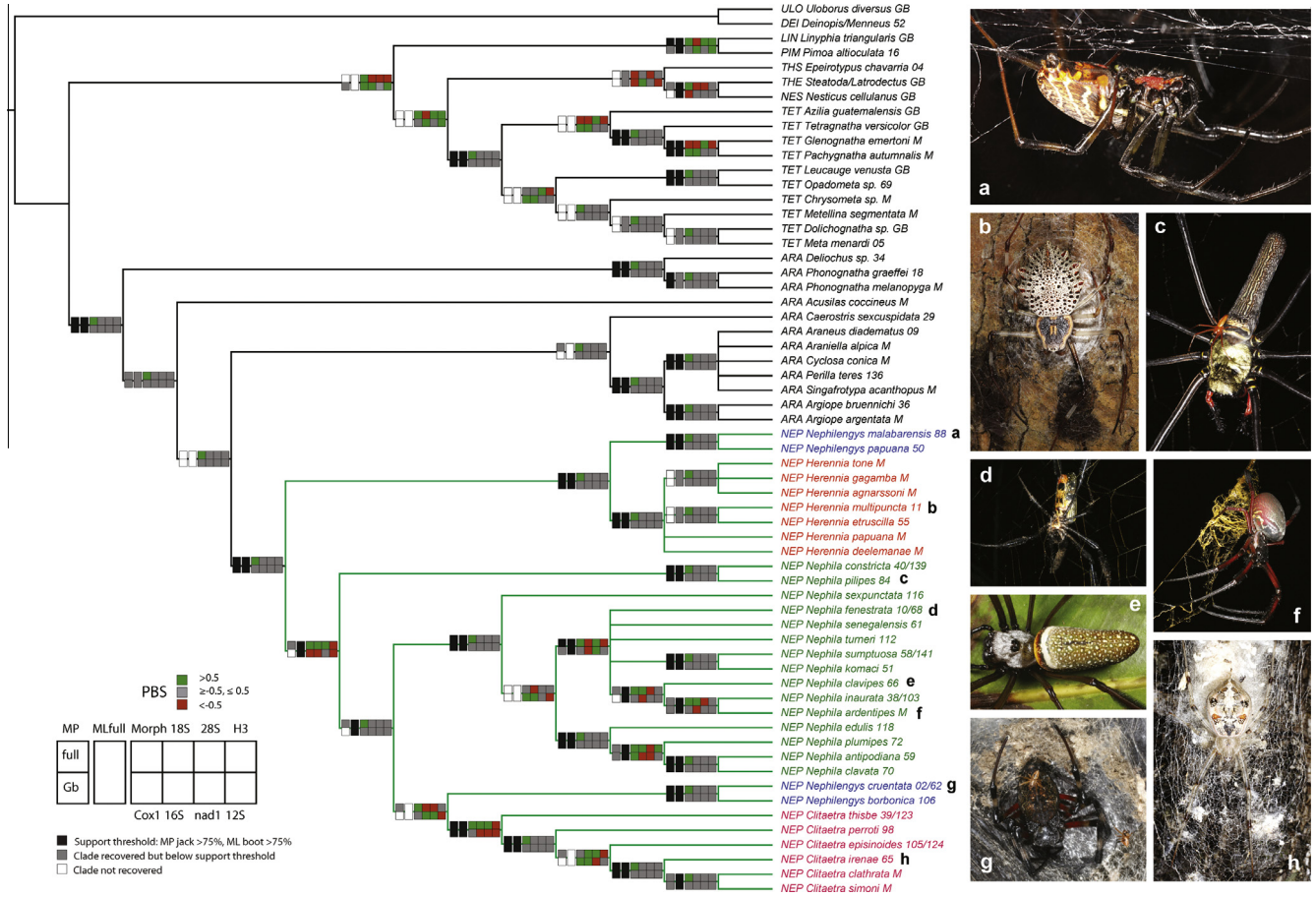

Fig. 1. A pictorial summary of nephilid phenotypic diversity (right, a–h), and a strict consensus of 36 trees resulting from parsimony analyses combining molecular markers (full matrix) with morphology (left). The three sets of squares on branches represent node supports from alternative analyses, as follows: the left set corresponds to the parsimony jackknife support for the full (above branch) and Gblocked (Gb, below) matrices, respectively. The middle bar shows maximum likelihood (ML) bootstrap support of the full matrix under the full codon partition scheme. The right set indicates the Bremer supports for the different partitions (PBS) on the reference tree: above branches, from left to right, values for morphology + behavior, followed by the Bremer support values for the nuclear genes and below branches for the mitochondrial genes. See legend for support thresholds. Terminals have the first three letters of current taxonomic familial placement (from bottom: NEP = Nephilidae, ARA = Araneidae, TET = Tetragnathidae, NES = Nesticidae, THE = Theridiidae, THS = Theridiosomatidae, PIM = Pimoidae, LIN = Linyphiidae, DEI = Deinopidae, ULO = Uloboridae). The ingroup, nephilid part of the tree is colored in green and the ingroup terminals are colored according to the accepted nomenclature prior to the classification changes in the current study. Terminals with original molecular data end with specimen codes (as in Table 1), those with data from GenBank end with GB, and those for which only morphological (and behavioral) data were used are labeled M.

As top invertebrate predators, golden orb weavers play important ecological roles in tropical and subtropical ecosystems. Furthermore, *Nephila* is a prominent biological model for a variety of questions in ecology (e.g. Miyashita, 1992a,b,c, 1993; Uetz et al., 2002; Zschokke et al., 2006), silk biology (e.g. Agnarsson et al., 2009; Blackledge, 2012; Blackledge et al., 2011; Blamires et al., 2012; Kummerlen et al., 1996; Liu et al., 2005; Madsen and Vollrath, 2000; Swanson et al., 2006a,b; Vollrath et al., 2001; Zhang et al., 2012), developmental biology (e.g. Higgins, 2000, 2002; Higgins and Goodnight, 2010, 2011; Higgins and Rankin, 1999; Kuntner et al., 2012b), functional morphology and physiology (e.g. Landolfa and Barth, 1996; Michalik and Rittschof, 2011; Uhl and Vollrath, 1998, 2000), biotechnology (e.g. Menassa et al., 2004), venom biology (e.g. Matsushita et al., 1995a,b; McCormick and Meinwald, 1993; Nishimaru et al., 2009) and behavioral ecology (e.g. Hesselberg, 2010; Higgins, 1992, 2006; Higgins and McGuinness, 1991; Kuntner et al., 2010a; Meraz et al., 2012; Nentwig and Spiegel, 1986; Rittschof, 2010, 2011, 2012; Rittschof et al., 2012; Rittschof and Ruggles, 2010; Tso et al., 2002, 2004, 2005, 2007). Behavioral studies also routinely use species of *Nephila* as models in the study of the mechanisms bearing on sexual selection (e.g. Christenson et al., 1985; Christenson and Cohn, 1988; Cohn, 1990; Cohn et al., 1988; Fromhage et al., 2007; Kuntner et al., 2009b,c, 2012a; Linn et al., 2007; Prenter et al., 2006; Schneider and Elgar, 2001, 2002, 2005; Schneider et al., 2000, 2001, 2005, 2008; Schneider and Michalik, 2011; Zhang et al., 2011). These cited works are merely a small selection; an exhaustive overview of *Nephila* biology and associated literature is beyond the scope of this paper.

For meaningful tests of evolutionary hypotheses, however, it is important to look beyond the model organism. An understanding of phylogenetic relationships of *Nephila* and relatives within the family Nephilidae at the species level is important, as is the understanding of variation in biological traits among species and clades. Nephilid spiders are morphologically quite diverse and the females range from small to huge, round to elongate, and cryptic to colorful, even showy (Fig. 1) and nephilid biology is also diverse. Recent taxonomic research on *Nephila* (Harvey et al., 2007; Kuntner and Agnarsson, 2011b; Kuntner and Coddington, 2009), *Nephilengys* (Kuntner, 2007; Kuntner and Agnarsson, 2011a), *Herennia* (Kuntner, 2005) and *Clitaetra* (Kuntner, 2006) has facilitated an understanding of nephilid phylogenetic structure at the species level (Kuntner and Agnarsson, 2009; Kuntner and Coddington, 2009; Kuntner et al., 2008a), and recent ecological and behavioral work has also focused on comparative biology of non-*Nephila* representatives of the family (Kralj-Fišer et al., 2011, 2012; Kralj-Fišer and Kuntner, 2012; Kuntner and Agnarsson, 2009; Kuntner et al., 2008b, 2009c; Lee et al., 2012; Li et al., 2012; Mendes et al., 2010; Năpăruş and Kuntner, 2012; Schuck-Paim, 2000; Schuck-Paim and Alonso, 2001).

The most serious hindrance to understanding the evolutionary history of Nephilidae, a seemingly old lineage (Kuntner, 2006), has been a poorly resolved nephilid phylogeny. Early attempts only dealt with nephilids at the genus/subfamily level (e.g. Hormiga et al., 1995), but were followed by more comprehensive species level phylogenies, all based on morphological and behavioral data (Kuntner, 2005, 2006, 2007; Kuntner et al., 2008a). The phylogeny of Kuntner et al. (2008a) established a monophyletic Nephilidae within the orb-weaving spider clade Araneoidea, and monophyletic nephilid genera *Nephila, Nephilengys, Herennia* and *Clitaetra*. Nevertheless, *a priori* morphological homology assessments are notoriously difficult and subjective, and several such assessments differed between the species level studies (Kuntner, 2005, 2006, 2007; Kuntner et al., 2008a) and previous work (Griswold et al., 1998; Hormiga et al., 1995; Scharff and Coddington, 1997), affecting the inferred topology. This may have been the reason that the nephilid species level phylogenies of Kuntner and colleagues were subsequently criticized for lack of objectivity (Dimitrov et al., 2009; Dimitrov and Hormiga, 2009). Rather than criticizing new homology assessments by simply restating earlier ones (e.g. Dimitrov et al., 2009), our approach is to test the existing topologies with independent data sources. These independently derived topologies can then, in turn, be used to evaluate morphological homology hypotheses.

Su et al. (2011) analyzed selected nephilid exemplars using molecular data from three genes (*coxI*, 16S, 18S). Focusing on Asian and Pacific *Nephila* species and their speciation patterns, their phylogeny only included 13 nephilid terminals with no representation of *Clitaetra* or Afrotropical *Nephilengys*. Nevertheless, the phylogeny of Su et al. (2011) provides a good point of comparison for our study. Other recent molecular phylogenies did not test any species level relationships within nephilids as their main focus was to resolve the relationships at higher taxonomic levels (Alvarez-Padilla et al., 2009; Blackledge et al., 2009; Dimitrov et al., 2012). We here focus on the first comprehensive, original molecular species-level phylogeny of nephilid spiders, to provide an independent test of family monophyly and phylogenetic affinity, and to test the monophyly of the four genera, the relationships among species and the evolutionary ages of different nephilid clades. Araneoid spiders appear to be older than previously thought (Dimitrov et al., 2012) and it has been suggested that *Nephila* was already present in the Jurassic (Selden et al., 2011), though the evidence is very weak (see also Sections 4 and 4.3). Fossils combined with molecular topologies may provide a new and powerful temporal perspective of nephilid evolution, which we also explore.

Furthermore, an independent and robust molecular phylogeny is needed not only to settle the disputed classification issues within Nephilidae (Alvarez-Padilla et al., 2009; Kuntner et al., 2008a), but primarily to offer a tool for evolutionary studies. For example, recent studies have investigated the (in)dependence of evolutionary size changes in males and females (Kuntner and Coddington, 2009), the variation in size with respect to phenotypic plasticity (Higgins et al., 2011), coevolutionary patterns of male and female genital complexity (Kuntner et al., 2009b), and the evolution of web architectures and functionality (Kuntner et al., 2010b). Building on the available phylogeny derived from morphological and behavioral data (Kuntner et al., 2008a), all of these studies lacked phylogenetic branch length information needed for more robust tests of evolutionary changes. Our phylogenetic results can now be used to retest and reinterpret key evolutionary events, from biogeography and speciation patterns, to web architecture and allometry, to intersexual genital coevolution, and ultimately, the size evolution in these sexually dimorphic animals.

2. Material and methods

2.1. Taxonomic sampling

2.1.1. Ingroup

Morphological and behavioral datasets of Kuntner and Agnarsson (2009), Kuntner and Coddington (2009) and Kuntner et al. (2008a) represent the most complete phylogenetic taxon sampling of nephilids to date. A major goal of our study was to mimic that taxon selection with recent material that could be used for DNA extraction, while also heeding the advances in *Nephila* and *Nephilengys* taxonomy since 2008. Our original ingroup taxonomic sample (Nephilidae in Table 1) is complete for extant *Nephila* with one terminal for each of the 14 species and for *Nephilengys* with 7 terminals and 6 species. Although Kuntner et al. (2008a) used 15 *Nephila* terminals at the time thought to represent all valid species, *Nephila ardentipes* Butler, 1876 from Rodrigues (Fig. 1f) has since

Table 1
Taxonomic, specimen, and gene information with GenBank accession numbers and with proportion of missing nucleotide data. Code refers to laboratory number used for specimen, n/a is used for those specimens whose sequences were gathered from GenBank, and asterisk marks those terminals used to complement taxon chimaeras. The taxonomic identification of nephilids followed recent revisions of *Herennia* (Kuntner, 2005), *Clitaetra* (Kuntner, 2006), *Nephilengys* (Kuntner, 2007; Kuntner and Agnarsson, 2011a) and *Nephila* (Kuntner, unpublished data; Kuntner and Coddington, 2009; Kuntner and Agnarsson, 2011b). The species *"Araneus" dimidiatus* is currently catalogued as such but in fact belongs in an as yet undescribed genus.

Code	Family	Genus	Species	Origin	12S	16S	*cox1-5′*	*cox1-3′*	28S	H3	18S	Missing data
n/a	Anapidae	*Elanapis*	*aisen*	GenBank	–	HM030401	HM030430	–	–	HM030437	–	0.684
77	Araneidae	*"Araneus"*	*dimidiatus*	Australia	KC848904	KC849109	KC849065	KC849065	KC848985	KC849024	KC848951	0.075
26	Araneidae	*Araneus*	*angulatus*	Spain	KC848903	KC849107	KC849063	KC849063	KC848983	KC849022	KC848949	0.076
9	Araneidae	*Araneus*	*diadematus*	Slovenia	–	KC849108	KC849064	KC849064	KC848984	KC849023	KC848952	0.141
36	Araneidae	*Argiope*	*bruennichi*	Slovenia	KC848902	KC849106	KC849062	KC849062	KC848982	KC849021	KC848941	0.075
29	Araneidae	*Caerostris*	*sexcuspidata*	South Africa	KC848905	KC849110	KC849066	KC849066	KC848986	KC849025	KC848948	0.076
25	Araneidae	*Cyrtophora*	*citricola*	Spain	KC848910	KC849115	KC849071	KC849071	–	KC849030	KC848942	0.191
34	Araneidae	*Deliochus*	*sp.*	Australia	–	KC849116	KC849072	KC849072	KC848990	KC849031	KC848943	0.137
22	Araneidae	*Larinioides*	*sclopetarius*	Slovenia	KC848915	KC849120	KC849077	KC849077	KC848995	KC849036	KC848950	0.075
136	Araneidae	*Perilla*	*teres*	China	KC848937	–	–	KC849102	KC849017	KC849058	KC848953	0.45
18	Araneidae	*Phonognatha*	*graeffei*	Australia	KC848938	KC849143	KC849103	KC849103	KC849018	KC849059	KC848944	0.085
42	Araneidae	*Zygiella*	*x-notata*	Croatia	KC848940	KC849145	KC849105	KC849105	KC849020	KC849061	KC848945	0.076
n/a	Cyatholipidae	*Alaranea*	*merina*	GenBank	–	AY230942	AY231022	–	AY231074	AY230982	AY230890	0.613
52	Deinopidae	*Menneus*	*camelus*	South Africa	KC848916	KC849122	KC849079	KC849079	KC848997	KC849038	KC848946	0.076
n/a	Holarchaeidae	*Holarchaea*	*sp.*	GenBank	–	–	–	–	EU302963	–	EU302915	0.719
n/a	Linyphiidae	*Linyphia*	*triangularis*	GenBank	–	AY078664	EU003292	–	EU003410	AY078702	EU003390	0.395
n/a	Malkaridae	*Malkara*	*sp.*	GenBank	–	–	–	–	EU302968	–	EU302920	0.719
n/a	Microphol commatidae	*Raveniella*	*peckorum*	GenBank	–	–	HM357565	–	EU303006	–	HM357532	0.548
n/a	Mimetidae	*Mimetus*	*sp.*	GenBank	–	FJ607463	FJ607574	–	FJ607538	FJ607612	FJ607500	0.486
n/a	Mysmenidae	*Maymena*	*ambita*	GenBank	–	GU456746	GU456876	–	–	GU456921	GU456765	0.64
124*	Nephilidae	*Clitaetra*	*episinoides*	Mayotte	–	–	–	–	KC848987	–	–	*
105	Nephilidae	*Clitaetra*	*episinoides*	Mayotte	KC848906	KC849111	KC849067	KC849067	–	KC849026	KC848979	0.076
65	Nephilidae	*Clitaetra*	*irenae*	South Africa	KC848907	KC849112	KC849068	KC849068	KC848988	KC849027	KC848980	0.073
98	Nephilidae	*Clitaetra*	*perroti*	Madagascar	KC848908	KC849113	KC849069	KC849069	KC848989	KC849028	KC848981	0.077
123*	Nephilidae	*Clitaetra*	*thisbe*	Sri Lanka	KC848909	–	–	–	–	–	–	*
39	Nephilidae	*Clitaetra*	*thisbe*	Sri Lanka	–	KC849114	KC849070	KC849070	–	KC849029	–	0.355
55	Nephilidae	*Herennia*	*etruscilla*	Indonesia	KC848912	KC849118	KC849074	KC849074	KC848992	KC849033	KC848956	0.073
11	Nephilidae	*Herennia*	*multipuncta*	Singapore	KC848913	KC849119	KC849075	KC849075	KC848993	KC849034	KC848958	0.072
113	Nephilidae	*Herennia*	*oz*	Australia	KC848914	–	KC849076	KC849076	KC848994	KC849035	KC848957	0.285
59	Nephilidae	*Nephila*	*antipodiana*	Malaysia	KC848917	KC849123	KC849080	KC849080	–	KC849039	KC848964	0.186
70	Nephilidae	*Nephila*	*clavata*	China	KC848919	KC849125	KC849082	KC849082	KC848999	KC849041	KC848965	0.075
66	Nephilidae	*Nephila*	*clavipes*	French Guiana	KC848918	KC849124	KC849081	KC849081	KC848998	KC849040	KC848970	0.086
139*	Nephilidae	*Nephila*	*constricta*	Cameroon	–	–	KC849087	KC849087	–	–	–	*
40	Nephilidae	*Nephila*	*constricta*	D. R. Congo	KC848920	–	–	–	KC849000	–	KC848962	0.419
118	Nephilidae	*Nephila*	*edulis*	Australia	KC848921	KC849126	KC849083	KC849083	KC849001	KC849042	KC848972	0.08
10	Nephilidae	*Nephila*	*fenestrata*	South Africa	KC848922	KC849127	KC849084	KC849084	KC849002	KC849043	KC848971	0.081
103*	Nephilidae	*Nephila*	*inaurata*	Madagascar	–	–	–	–	KC849003	–	–	*
38	Nephilidae	*Nephila*	*inaurata*	Madagascar	KC848923	KC849128	KC849085	KC849085	–	KC849044	KC848966	0.077
51	Nephilidae	*Nephila*	*komaci*	South Africa	KC848924	KC849129	KC849086	KC849086	–	–	–	0.433
84	Nephilidae	*Nephila*	*pilipes*	Indonesia	KC848925	KC849130	KC849088	KC849088	KC849004	KC849045	KC848963	0.073
72	Nephilidae	*Nephila*	*plumipes*	Australia	–	KC849131	KC849089	KC849089	KC849005	KC849046	KC848973	0.14
61	Nephilidae	*Nephila*	*senegalensis*	South Africa	KC848926	KC849132	KC849090	KC849090	KC849006	KC849047	KC848968	0.217
116	Nephilidae	*Nephila*	*sexpunctata*	Brazil	KC848927	KC849133	KC849091	KC849091	–	KC849048	KC848969	0.19
58*	Nephilidae	*Nephila*	*sumptuosa*	Oman	KC848928	–	–	–	–	–	–	*
141	Nephilidae	*Nephila*	*sumptuosa*	Yemen	–	KC849134	KC849092	KC849092	KC849007	–	KC848967	0.154
112	Nephilidae	*Nephila*	*turneri*	Ghana	–	KC849135	KC849093	KC849093	KC849008	KC849049	KC848974	0.141
106	Nephilidae	*Nephilengys*	*borbonica*	Reunion	KC848929	KC849136	KC849094	KC849094	KC849009	KC849050	–	0.245
2*	Nephilidae	*Nephilengys*	*cruentata*	South Africa		KC849137	–	–	KC849010	KC849051	KC848975	*
96	Nephilidae	*Nephilengys*	*cruentata*	Brazil	KC848931	–	KC849096	KC849096	KC849011	KC849052	KC848976	0.29
62	Nephilidae	*Nephilengys*	*cruentata*	South Africa	KC848930	–	KC849095	KC849095	–	–	–	0.077
107	Nephilidae	*Nephilengys*	*dodo*	Mauritius	KC848932	KC849138	KC849097	KC849097	KC849012	KC849053	KC848978	0.074
108*	Nephilidae	*Nephilengys*	*livida*	Mayotte	KC848933	–	–	–	–	–	–	*

Table 1 (*continued*)

Code	Family	Genus	Species	Origin	12S	16S	*cox1-5′*	*cox1-3′*	28S	H3	18S	Missing data
76	Nephilidae	*Nephilengys*	*livida*	Madagascar	–	KC849139	KC849098	KC849098	KC849013	KC849054	KC848977	0.077
88	Nephilidae	*Nephilengys*	*malabarensis*	Singapore	KC848934	KC849140	KC849099	KC849099	KC849014	KC849055	KC848959	0.078
50	Nephilidae	*Nephilengys*	*papuana*	Australia	KC848935	KC849141	KC849100	–	KC849015	KC849056	KC848960	0.213
n/a	Nesticidae	*Nesticus*	*cellulanus*	GenBank	–	EU746444	EU746435	–	AF124961	–	AF005447	0.557
n/a*	Nicodamidae	*Oncodamus*	*decipiens + bidens*	GenBank	–	EU003274	FJ949011	–	FJ949971	FJ949048	FJ948887	0.534
16	Pimoidae	*Pimoa*	*altioculata*	Canada	KC848939	KC849144	–	KC849104	KC849019	KC849060	KC848947	0.234
n/a	Symphytognathidae	Unknown	*sp.*	GenBank	GU456741	–	GU456911	–	–	GU456964	GU456814	0.67
n/a	Synotaxidae	*Synotaxus*	*sp.*	GenBank	–	AY230943	AY231026	–	AY231076	AY230986	AY230894	0.611
n/a	Tetragnathidae	*Arkys*	*cornutus*	GenBank	–	FJ607448	FJ607556	–	FJ607521	FJ607595	FJ607482	0.348
n/a	Tetragnathidae	*Azilia*	*guatemalensis*	GenBank	–	EU003262	EU003280	–	EU003399	EU003313	EU003373	0.569
n/a	Tetragnathidae	*Dolichognatha*	*sp.*	GenBank	–	–	EU003285	–	EU003405	EU003317	EU003346	0.487
n/a	Tetragnathidae	*Leucauge*	*venusta*	GenBank	EU003238	FJ607457	FJ607568	–	FJ607533	FJ607606	FJ607494	0.287
5	Tetragnathidae	*Meta*	*menardi*	Slovenia	–	KC849121	KC849078	KC849078	KC848996	KC849037	KC848954	0.138
69	Tetragnathidae	*Opadometa*	*sp.*	Singapore	KC848936	KC849142	KC849101	KC849101	KC849016	KC849057	KC848955	0.077
n/a	Tetragnathidae	*Tetragnatha*	*versicolor*	GenBank	EU003246	FJ525350	FJ525317	–	EU003429	FJ525336	FJ525387	0.244
n/a	Theridiidae	*Anelosimus*	*studiosus*	GenBank	–	EF050157	EF050320	–	EF050260	EF050367	EF050200	0.371
n/a	Theridiidae	*Latrodectus*	*geometricus*	GenBank	–	FJ60745	FJ607567	–	FJ607532	FJ607605	FJ607493	0.435
4	Theridiosomatidae	*Epeirotypus*	*chavarria*	Costa Rica	KC848911	KC849117	KC849073	KC849073	KC848991	KC849032	KC848961	0.073
n/a	Theridiosomatidae	*Theridiosoma*	*gemmosum*	GenBank	HM030400	AY230939	HM030436	–	–	HM030443	HM030417	0.451
n/a	Uloboridae	*Uloborus*	*diversus*	GenBank	–	FJ525362	FJ525329	–	–	FJ525345	FJ525399	0.455

then been shown to represent a morphotype nested within *N. inaurata* (Walckenaer, 1841) (Kuntner and Agnarsson, 2011b). Conversely, Western Indian Ocean populations of *Nephilengys borbonica* (Vinson, 1863) s.l. have been shown to represent three valid species instead of one: *N. livida* (Vinson, 1863) in Madagascar and Comoros, *N. borbonica*, which rediagnosed is limited to Réunion, and *N. dodo* Kuntner & Agnarsson, 2011 endemic to Mauritius (Kuntner and Agnarsson, 2011a). Since the known range of *Nephilengys cruentata* (Fabricius, 1775) spans Africa and South America (Kuntner, 2007), the species is represented here by an exemplar from each continent. There have been no changes in *Herennia* and *Clitaetra* taxonomy since their revisions (Kuntner, 2005, 2006), but the limited availability of these taxa in recent collections precluded a thorough representation in the current study: while *Clitaetra* is represented by all but two species (the western African *C. clathrata* Simon, 1907 and *C. simoni* Benoit, 1962 were unavailable), *Herennia* is represented by only three out of 11 described species. Seven ingroup terminals were complemented with conspecific sequences from multiple individuals, and these represent chimaeras, marked with asterisk in Table 1. Thus, in total, 28 ingroup terminals representing 27 described nephilid species were included for molecular analyses of the full matrix (see below). All ingroup sequence data are original, with GenBank accession numbers as in Table 1.

2.1.2. Outgroups

The precise placement of Nephilidae within the Araneoidea, and thus its familial affiliation has been contentious. Traditionally, *Nephila* and relatives were placed within Araneidae, although cladistic treatments in the 1990s, based on relatively few morphological and behavioral characters, recovered them as basal tetragnathids (Hormiga et al., 1995), and thus they were at the time considered members of Tetragnathidae (for a review of this taxonomic history, see Kuntner et al., 2008a). Newer cladistics analyses, based on revised taxonomy and morphological and behavioral homologies did not corroborate the tetragnathid phylogenetic position of nephilids (Kuntner, 2005, 2006, 2007; Kuntner et al., 2008a) but subsequent morphological work restated the "tetragnathid hypothesis" by using legacy morphological homologies (Dimitrov and Hormiga, 2009). It is worth noting that the tetragnathid hypothesis has not been supported by any molecular phylogenetic study of araneoid interfamilial relationships (Alvarez-Padilla et al., 2009; Blackledge et al., 2009; Dimitrov et al., 2012). Hence, in order to retest nephilid monophyly and its controversial phylogenetic affinities, we used a wide selection of outgroups spanning most families currently considered to be araneoids (Blackledge et al., 2009; Dimitrov et al., 2012; Griswold et al., 1998), but emphasizing both tetragnathids and araneids. All trees were rooted with a non-araneoid representative of the genus *Menneus* (Deinopidae) (Table 1), the likely sister group of Araneoidea (Blackledge et al., 2009; Coddington, 1986a,b; Dimitrov et al., 2012; Griswold et al., 1998). Table 1 lists the sources for the outgroups (GenBank versus original data) and proportions of available sequence data for each taxon.

2.2. Molecular procedures

We isolated DNA from spider muscles or abdomens using the GenElute Mammalian Genomic DNA Miniprep kit (Sigma, St. Louis, USA) following the Mammalian Tissue protocol. We amplified fragments of five mitochondrial and three nuclear loci, which have, because of universal primer availability, become the standard genes of choice in spider phylogenetics. Most PCR reactions had a total volume of 25 μl, consisting of 12.95 μl of dd H_2O, 5.0 μl of 5× PCR buffer GoTaqFlexi (Promega), 2.25 μl of $MgCl_2$ (25 mM, Promega), 2.5 μl of dNTP Mix (2 μM each, Biotools), 0.5 μl of each forward

and reverse 20 μM primer, 0.15 μl of bovine serum albumin (Promega, 10 mg/ml), 0.15 μl of 5 U GoTaqFlexi Polimerase and 1 μl of DNA. Amplificaton was performed on a "2720 Thermal Cycler" (Applied Biosystems, USA) and on a "Mastercycler® ep" (Eppendorf, Germany). For each gene fragment we used different protocols with varying annealing temperatures and PCR cycles. The majority of samples were processed using a touch up protocol.

We obtained ~1 kb fragment of the mitochondrial cytochrome c oxidase subunit I gene (*coxI*) by using two primer combinations: the forward "LCO 1490" (GGTCAACAAATCATAAAGATATTGG) (Folmer et al., 1994) with the reverse "HCO 2198" (TAAACTTCAGGGTGACCAAAAAATCA) (Folmer et al., 1994), and the forward "Jerry" (CAACATTTATTTTGATTTTTTGG) (Simon et al., 1994) with the reverse "Maggie" (GGATAATCAGAATATCGTCGAGG) (Hedin and Maddison, 2001). For the first primer combination "LCO 1490" and "HCO 2198" fragments were amplified using a touch up program. PCR cycling conditions were 96 °C for 10 min, followed by 20 cycles of 94 °C for 1.5 min, 48–58 °C for 2 min, 72 °C for 2 min, followed by 15 cycles of 94 °C for 1.5 min, 52 °C for 1.5 min, 72 °C for 2 min and a final extension period of 72 °C for 3 min. "Jerry" + "Maggie" fragments were also amplified using a touch up program with PCR cycling conditions 94 °C for 5 min, followed by 20 cycles of 94 °C for 1.5 min, 48–55 °C for 2 min, 72 °C for 3 min, followed by 18 cycles of 94 °C for 1.5 min, 48 °C for 2 min, 72 °C for 3 min and a final extension of 72 °C for 3 min.

To amplify ~1 kb fragment of parts of three mitochondrial markers, the large ribosomal subunit gene 16S rRNA (16S), the tRNA-leu (L1) and the complex 1 NADH-ubiquinone oxidoreductase (*nad1*) we used the forward primer "16S-ar" (CGCCTGTTTATCAAAAAC) (Palumbi et al., 1991) and the reverse primer "SPIDND1" (TCRTAAGAAATTATTTGAGC) (Hedin, 1997). Fragments were amplified using a touch up program with PCR cycling conditions 94 °C for 5 min, followed by 20 cycles of 94 °C for 1 min, 45–55 °C for 1 min 50 s, 72 °C for 2.5 min, followed by 17 cycles of 94 °C for 1.5 min, 52 °C for 1 min 50 s, 72 °C for 2.5 min and a final extension of 72 °C for 10 min.

For the ~270 bp mitochondrial small subunit ribosomal gene 12S rRNA (12S) we used the forward primer "St-L" (GGTGGCATTTTATTTTATTAGAGG) (Croom et al., 1991) with the reverse primer "12S-bi-H" (AAGAGCGACGGGCGATGTGT) (Simon et al., 1994). Fragments were amplified using a thermal cycle of 94 °C for 3 min, followed by 35 cycles of 94 °C for 45 s, 46 °C for 30 s, 72 °C for 1 min and a final extension of 72 °C for 3 min.

For the ~810 bp nuclear small subunit ribosomal gene 18S rRNA (18S) we used the forward primer "18S-lev2 (18s ai)" (CCTGAGAAACGGCTACCACATC) (Whiting et al., 1997) with the reverse "18S-b0" (GTTTCAGCTTTGCAACCAT) (Whiting et al., 1997). Fragments were amplified using a touch up program with PCR cycling conditions of 94 °C for 5 min, followed by 20 cycles of 94 °C for 55 s, 49–59 °C for 1 min 50 s, 72 °C for 2 min, followed by 15 cycles of 94 °C for 55 s, 56 °C for 1.5 min, 72 °C for 2 min and a final extension of 72 °C for 10 min.

For the ~700 bp nuclear large subunit ribosomal gene 28S rRNA (28S) we used the forward primer "28S-A" (GACCCGTCTTGAAACACGGA) (Whiting et al., 1997) with the reverse "28S-rd5b" (CCACAGCGCCAGTTCTGCTTAC) (Whiting, 2002). PCR cycling conditions were 96 °C for 7 min, followed by 20 cycles of 96 °C for 45 s, 62–52 °C for 45 s, 72 °C for 1 min, followed by 15 cycles of 96 °C for 45 s, 52 °C for 45 s, 72 °C for 1 min and a final extension of 72 °C for 10 min.

Finally, for the ~325 bp nuclear protein coding histone 3 subunit A (H3) we used two primer combinations (Colgan et al., 1998), the forward "H3aF1" (ATGGCTCGTACCAAGCAGACVGC) with the reverse "H3aR1" (ATATCCTTRGGCATRATRGTGAC), and the forward primer "H3aF2" (ATGGCTCGGTACCAAGCAGAC) with the reverse "H3aR2" (ATRTCCTTGGGCATGATTGTTAC). PCR cycling conditions were 94 °C for 3 min, followed by 20 cycles of 94 °C for 45 s, 42–50 °C for 1.5 min, 72 °C for 1.5 min, followed by 15 cycles of 94 °C for 45 s, 46 °C for 1.5 min, 72 °C for 1.5 min and a final extension of 72 °C for 10 min.

PCR products were subjected to electrophoresis on ethidium bromide stained agarose gel (1%) and photographed under UV light, then purified using a membrane system (Millipore Montagne, Bedford, USA). Products were sequenced by Macrogen services (www.macrogen.com). Chromatograms were combined and edited using ChromasPro 1.5 (Technelysium Pty. Ltd. 2009) and Mega 5.05 (Tamura et al., 2011). Preliminary alignments were done using MUSCLE (Edgar, 2004a,b). All sequences were checked by blasting and screening protein coding genes for internal stop codons, then submitted to GenBank (for accession numbers, see Table 1).

2.3. Alignments

Unlike protein coding genes, the ribosomal gene fragments showed unequal lengths and were aligned with the online version of the automatic aligner MAFFT v.6 (http://mafft.cbrc.jp/alignment/server/), using secondary structure of RNA information during the alignment process (the Q-INS-i strategy) and default values.

The retrieved alignments of the nuclear ribosomal genes (18S, 28S) included regions with a highly unequal distribution of indels, especially in nephilids. Alternative alignment methods and algorithms for detecting and removing regions of ambiguous alignment were conducted to assess the impact of ambiguously aligned positions on the inferred relationships. First, new matrices were constructed by removing positions of uncertain alignment with the help of the computer program Gblocks v.0.91b using default values (stringent). Second, we reanalyzed the full dataset with the program SATé v.2.2.3 (Liu et al., 2012) to assess the effect of using alternative alignment methods. This program implements an iterative procedure that alternates rounds of automatic alignment construction with tree inference to improve the final alignment and return the best ML tree. SATé is a highly efficient approach that produces much more accurate alignments and trees than standard two-phase approaches on highly divergent, gappy sequences (Liu et al., 2009). We used MAFFT as initial aligner, MUSCLE (Edgar, 2004a,b) as merger and FASTTREE (Price et al., 2010) as tree estimator, with an extra search with RAxML. We ran the SATé-II-fast algorithm with default parameters.

A combination of Mesquite (Maddison and Maddison, 2012) and WINCLADA (Nixon, 2002) was used for concatenating individual gene fragments into a single matrix, available as electronic Supplementary file (ESM A).

2.4. Morphology and behavior combined with molecular data

We explored whether the phylogenetic signal in our molecular dataset was sensitive to combined analyses. Thus, we tested if the nephilid morphological and behavioral dataset (Kuntner et al., 2008a) might impact the molecular results from the current study. We used all taxa from the morphological and behavioral matrix, including those for which we lacked molecular data, but omitted those taxa for which we lacked morphology and behavior. We thus combined those terminals from the molecular and morphological datasets that belong to the same species and genera, then combined the following terminals into chimaeras: *Latrodectus geometricus* C.L. Koch, 1841 (our study) with *Steatoda* (both Theridiidae), *Menneus camelus* Pocock, 1902 (our study) with *Deinopis* (both Deinopidae), and *Nephilengys livida* (our study) with *N. borbonica*. The morphological and behavioral matrix with 231 characters (Kuntner et al., 2008a) is complemented here with the published data updates for *Clitaetra* (Kuntner and Agnarsson, 2009) and for *Nephila komaci* (Kuntner and Coddington, 2009). This updated morphological and

behavioral matrix, superseding the prior ones (Kuntner and Agnarsson, 2009; Kuntner and Coddington, 2009; Kuntner et al., 2008a), and the combined matrix with molecular data, are both available as electronic Supplementary files (ESM B, C).

2.5. Phylogenetic inference

2.5.1. Model based methods

Maximum likelihood analyses were conducted with RAxML v.7.2.8 (Stamatakis, 2006). The search strategy consisted of the rapid bootstrapping algorithm followed by the search of the best scoring tree (-f a algorithm). We used the extended majority-rule consensus tree criterion to decide upon the optimal number of bootstraps (i.e. autoMRE). Preliminary partitioned analyses were conducted to assess congruence across gene fragments. The best partitioning scheme and corresponding substitution models were selected with the help of the program PARTITIONFINDER (Lanfear et al., 2012). We defined eight alternative partition schemes, including the use of unlinked models for each gene and codon position in protein coding genes (full codon partition scheme), the unlinked models for each gene and for the combined first + second and the third codon positions in protein coding genes (simple codon partition scheme) and the unlinked models for each gene (gene partition scheme). All implemented metrics selected the full codon partition as the best scheme. Combined analyses of the morphological and molecular data sets were conducted by defining a specific multi-state character model (-K GTR) for the morphological partition and a general MULTICAT model for the whole dataset.

In addition, RAxML was used to obtain the likelihood values per site under alternative topological hypotheses (only for the molecular data set, see Section 3). The *p*-values of alternative hypotheses under the Approximately unbiased test (AU) (Shimodaira, 2002) were calculated with the program CONSEL (Shimodaira and Hasegawa, 2001), using the multi-scale bootstrap techniques.

Bayesian analyses were conducted with the parallel version of the program MrBayes v3.1.2 (Ronquist and Huelsenbeck, 2003) run remotely at the Bioportal computer resources of the University of Oslo (http://www.bioportal.uio.no). Analyses were conducted under the preferred partition scheme (full partition) as well as under two additional schemes (simple codon and gene) to investigate the effect of using simpler models. Four independent runs with eight simultaneous MCMC (Markov Chain Monte Carlo) chains (temperature = 0.15), each starting with random starting trees, were performed for 50 million generations. The standard deviation of the split frequencies between runs (<0.01) and the effective sample size (ESS, as measured by the program TRACER 1.5; Rambaut and Drummond, 2007), were monitored to ensure stationarity, convergence and correct mixing of the chains. TRACER was further used to select the number of generations discarded as burn-in.

2.5.2. Parsimony

Parsimony analyses were conducted with the program TNT v.1.1 (Goloboff et al., 2008) using a heuristic RAS search with 1000 starting Wagner trees and TBR branch swapping, keeping 10 trees per replicate, with an additional round of swapping on the shortest trees found. Supports were inferred based on 1000 jack-knifed matrices. Analyses were run on the morphological, the molecular, and the combined datasets, using both the full and the gblocked matrices. Approximate partitioned Bremer supports (PBS) were estimated using a TNT tailored script (Pena et al., 2006).

2.6. Estimating the time frame of nephilid diversification

The deep differences observed in branch lengths across taxa and genes suggested relaxing the assumption of a strict clock. Molecular dating was thus conducted under a Bayesian framework using relaxed clock methods as implemented in BEAST v1.7.2 (Drummond et al., 2012). We combined fossil evidence with informed priors on the mitochondrial substitution rate to calibrate the relaxed clock and to estimate divergence times.

Several shortcuts were implemented to simplify calculations. First, analyses were conducted on the molecular data of the combined data matrix to reduce the number of irrelevant taxa and to constrain certain clades recovered as monophyletic in the combined analyses. Second, the number of model partitions was reduced to seven by only considering the individual genes (16S and L1 were combined in a single partition). The best fitting evolutionary models for each gene were determined with PARTITIONFINDER (Lanfear et al., 2012). Preliminary runs showed double peaks in the marginal density of the I and G parameters in 16S+L1, *nad1* and 12S genes, suggesting some kind of negative interaction between the two parameters. Consequently we simplified the model in these genes to include only the gamma parameter. Third, all mitochondrial genes were linked in a single clock, while each nuclear gene was assigned its own. Relaxed lognormal clocks were used for each of the partitions except H3, for which a strict clock was defined, as indicated by the low values of the ucld.stdev parameter in preliminary runs. Finally, all genes were enforced to support the same underlying tree topology (i.e. concatenated analysis). We conducted two independent runs of 50 million generations for each calibration scheme (see below) to assess chain convergence. All analyses were run remotely at the Bioportal computer resources of the University of Oslo. The correct mixing of each MCMC chain and the burn-in was visualized with TRACER. The independent runs under each calibration scheme were combined and trees summarized using the BEAST-accompanying LogCombiner and TreeAnnotator.

2.6.1. Calibration points

Absolute divergence times were estimated by incorporating fossil taxa as minimum bounds for selected lineages. A number of araneoid fossil species have been described and used in previous analyses to calibrate trees (Dimitrov et al., 2012). We examined the primary literature for these fossils with the purpose of corroborating the morphological evidence for their hypothesized placement. Although all of them are plausibly araneoids, we found most of the evidence for their precise familial placement unconvincing except for a linyphiine species described from Lebanese amber (125–135 Ma) (Penney and Selden, 2002), which we used to calibrate the split of Linyphiidae and its sister group Pimoidae to 125 Ma. We used all other ambiguously placed araneoid fossils to set the minimal age for araneoids, an assumption that places fewer constraints on the analysis than assigning minimal ages to doubtful families. Therefore, *Macryphantes cowdeni* Selden, 1990 and *Huegina diazromerali* Selden and Penney, 2003, both from Lower Cretaceous deposits in Spain (125–135 Ma) (Selden, 1990; Selden and Penney, 2003) set the minimum age for Araneoidea, rather than for their originally proposed family Tetragnathidae. Similarly, *Mesozygiella dunlopi* Penney and Ortuno, 2006 described from Lower Cretaceous amber from Spain (115–121 Ma) (Penney and Ortuno, 2006) sets the minimum bound for Araneoidea, not the Araneidae stem group to 115 Ma, because, in our opinion, it is neither an araneid nor close to *Zygiella*. In addition, the fossil genus *Jurararaneus*, described from the lower Middle Jurassic (Eskov, 1984) has been considered as the oldest araneoid, although it cannot be placed in any of the extant families and thus sets the minimum divergence date for the root of our tree (the split of Araneoidea from its sister taxon, Deinopoidea) to 176 Ma.

Morphological evidence placing the nephilid fossils described from Baltic Amber (Wunderlich, 2004) is inconclusive, except in the case of *Palaeonephila*, which exhibits a striated cheliceral

condyle (boss), as do all extant nephilids (Kuntner et al., 2008a). As *Palaeonephila* is distinct from all extant nephilid clades, we therefore use its age (40 Ma) as a minimum bound for the stem group of nephilids. The described species of Dominican amber *Nephila* (Wunderlich, 1986) indeed resemble the extant forms, and thus can be used to assign a minimum bound (16 Ma) to the stem group of *Nephila* (see below).

Recently, *Nephila jurassica* Selden, Shih and Ren, 2011 was described from the Middle Jurassic (approx. 165 Ma) strata of Daohugou, Inner Mongolia, China (Selden et al., 2011). Further examinations of this and putatively related specimens subsequent to this publication suggested that *N. jurassica* may be cribellate (P. Selden, 2012 presentation, European Congress of Arachnology, Ljubljana, Slovenia). Instead of taking that as positive evidence that the species does not belong to *Nephila*, a group nesting within the ecribellate Araneoidea, the authors reiterated the placement of the species as the sole cribellate *Nephila*, and indeed araneoid—a highly dubious assertion— requiring a novel origin of the cribellum. In our opinion, this species differs dramatically from the extant *Nephila* in many obvious traits, such as habitus, distribution of femoral tufts, location and morphology of the 'sustentaculum', and male palpal morphology (unknown in original description but reported since). At the same time, the *N. jurassica* fossil does not reveal a key nephilid synapomorphy, a striated cheliceral boss, and we thus treat this fossil accordingly (see below). However, to test the plausibility of the assertion that *Nephila* origins may trace back to the Jurassic (165 Ma), additional calibration points were included based on three different scenarios. The first scenario sets the minimum age of the stem group of *Nephila* to the age of the reported fossil (*Nephila* = 165 Ma min). The second scenario treats *N. jurassica* as the stem nephilid (nephilids = 165 Ma min). Finally, under the third scenario *N. jurassica* is reinterpreted as to belong to the stem group of Orbiculariae, and hence we used the additional fossil evidence to provide minimum bounds for the stem nephilids (40 Ma), and the stem *Nephila* (16 Ma).

Fossil information was incorporated as priors to account for data uncertainty (Ho and Phillips, 2009). Calibration points were assigned an exponential distribution, with starting value corresponding to the minimum bound and mean values such as the 95% interval including ages 10% older than the minimum bound (Hipsley et al., 2009). A preliminary tree including all time constraints was obtained with STARTTREE (Heath, 2012) and specified as starting tree in the BEAST runs.

2.6.2. Substitution rates

By implementing the protocol proposed by Cicconardi et al. (2010), we first confirmed that the mitochondrial substitution rates of the orbicularian taxa including nephilids fit the values reported by Bidegaray-Batista and Arnedo (2011). These autors recently provided spider-specific substitution rates estimates for mitochondrial genes (mean = 0.0112, SD = 0.001), based on the well-resolved geochronology of the Mediterranean basin. In short, two chains of 10^7 million generations were run with BEAST on a data matrix combining the mitochondrial sequences of orbicularian taxa included in the present study with those of the family Dysderidae from Bidegaray-Batista and Arnedo (2011) and arbitrarily fixing the substitution rate to 1. A single GTR+I+G model and relaxed clock were defined for the combined matrix to speed up calculations. Average and standard deviation of the substitution rate per branch obtained from the inferred ultrametric tree were estimated and compared for those families represented by several terminals, namely Dysderidae, Tetragnathidae, Araneidae, and Nephilidae.

The values estimated for the orbicularian families overlapped with those of the family Dysderidae (electronic Supplementary file ESM D), and hence the substitution rates reported for the latter can be safely implemented to estimate divergence times in orbicularians. Therefore, along with the calibration point information, the mitochondrial substitution rate parameter (ucld.mean) was assigned a normal prior with mean and SD as stated above (mean = 0.0112, SD = 0.001). Substitution mean rates of the nuclear genes were assigned uniform flat priors, with starting values an order of magnitude slower than the reported mitochondrial rate.

3. Results

The full, concatenated molecular data matrix (hereafter referred to as the full matrix) included 65 terminals (28 nephilids) and 4197 characters (*cox1*: 1244 bp, 16S+L1: 591 bp, *nad1*: 362 bp, 12S: 279 bp, 18S: 809 bp, 28S: 572 bp, H3: 340 bp). Removal of positions of uncertain alignment with Gblocks reduced characters of ribosomal genes to 294, 186, 307 and 309 bp, for 16S+L1, 12S, 18S and 28S, respectively (hereafter referred to as the Gblocked matrix). The combined morphological and molecular data set included 61 terminals (sequenced taxa with no morphology scored were removed while 18 taxa scored for morphology but without sequence data were retained), and 4428 characters, of which 231 were morphological and behavioral characters (Kuntner et al., 2008a), or 3273 characters when uncertain alignment positions were removed.

Nuclear ribosomal genes were observed to have an accelerated rate of evolution in nephilid lineages (Fig. 3), and thus we opted for a conservative approach and treated gaps as missing data in all analyses to ameliorate the impact of highly variable, gappy positions on the phylogenetic analyses.

Results of the phylogenetic analyses under the different inference methods and datasets are summarized in Figs. 1 and 2. Parsimony analyses of the full, concatenated matrix yielded 6 trees of 15,504 steps and the Gblocked, concatenated matrix resulted in 6 trees of 11,207 steps. The full, combined morphology and molecular matrix yielded 36 trees of 12,631 steps and the Gblocked, combined data matrix yielded 124 trees of 9227 steps. Maximum likelihood and Bayesian analyses, under the three-partition schemes, converged on similar topologies regarding Nephilidae and close relatives, with only slight differences in support values. The tree recovered with the iterative approximation to alignment and tree estimation implemented in SATé (not shown) closely resembled the best tree obtained by RAxML with the standard single MAFFT alignment, and the only differences were at nodes with low support in the RAxML tree.

All analyses recovered monophyletic Nephilidae as circumscribed in recent literature (Kuntner et al., 2008a), containing the species from traditional genera *Nephila, Nephilengys, Herennia* and *Clitaetra*, and the support for this node is strong (Figs. 1 and 2). Although the outgroup relationships, and thus the phylogenetic affinities of nephilids within Araneoidea vary among the results from different phylogenetic approaches, these are mostly poorly supported and remain ambiguous. The monophyly of Nephilidae + "Zygiellidae" + Araneidae is recovered in almost all analyses (Figs. 1 and 2), refuting the tetragnathid hypothesis of nephilid affiliation. In some analyses (for example, parsimony, ML full and Bayesian full under full and simple codon partition), this clade also includes non-araneid terminals that are apparently difficult to place using the relatively sparse sequence data (Table 1), the theridiosomatid *Theridiosoma* and the micropholcommatid *Raveniella*. A comparison of relative branch lengths for each gene tree (Fig. 3) reveals that while the rates of evolution are comparable for the outgroup and the ingroup in the case of mitochondrial genes and the nuclear H3, the evolution rates in the nuclear ribosomal genes (18S and 28S) are much higher in nephilids compared with the outgroups. Since nuclear ribosomal gene trees also

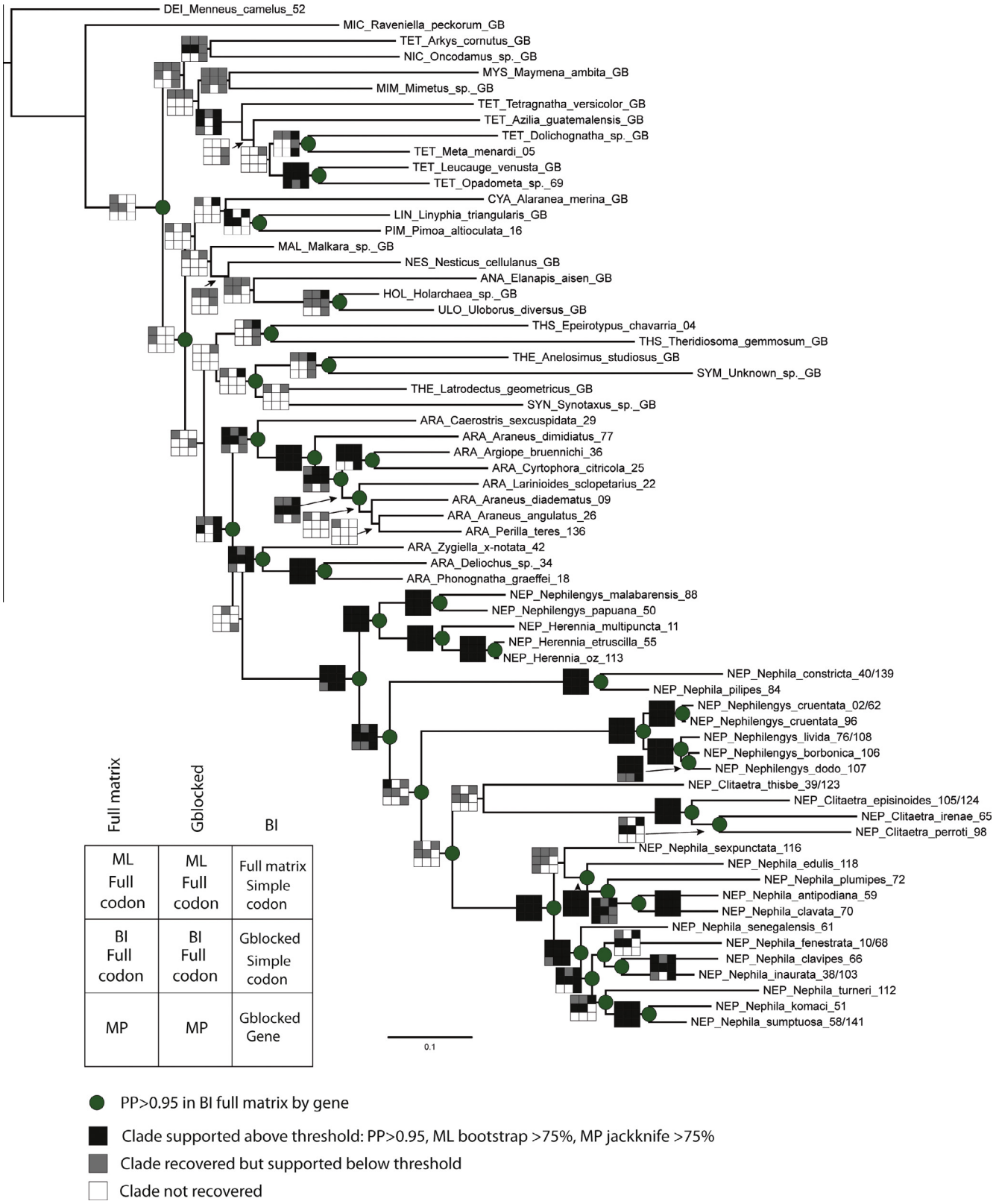

Fig. 2. Summary results from the analyses of the molecular matrices. The topology is from the Bayesian analysis of the full matrix partitioned by gene, with posterior probability values above 95% labeled with green dots at nodes. The nine squares on branches summarize the results of the alternative analyses using maximum likelihood (ML), maximum parsimony (MP) and Bayesian inference (BI) on different matrices and partition schemes (key in upper part of legend). Bar colors are indicative of clade support (key in lower part of legend) with solid squares indicating high support, gray squares indicating low support, and empty squares indicating a clade not recovered. Terminal legend as in Fig. 1, but with additional families (from top: MIC = Micropholcommatidae, NIC = Nicodamidae, MYS = Mysmenidae, MIM = Mimetidae, CYA = Cyatholipidae, MAL = Malkaridae, ANA = Anapidae, HOL = Holarchaeidae, SYM = Symphytognathidae, SYN = Synotaxidae). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

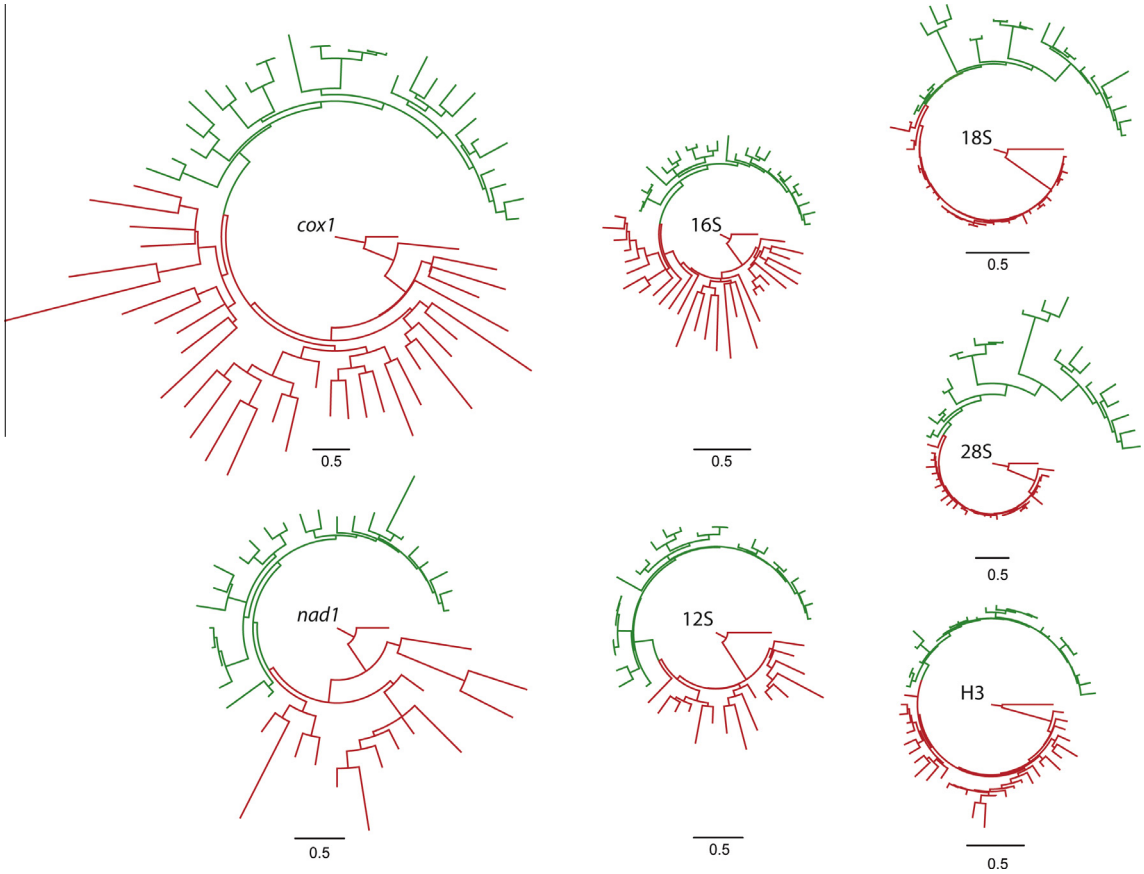

Fig. 3. Tree shapes for seven genes obtained with ML (best tree out of 100 replicates) with ingroup in green and outgroups in red. Note that for the two nuclear ribosomal genes (18S and 28S) the ingroup branch lengths are disproportionately long. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 2
Results of the topological tests conducted on the full, concatenated molecular data matrix under alternative topological constraints. *P*-values for **au** (approximately unbiased test), **kh** (Kishino–Hasegawa test), **sh** (Shimodaira–Hasegawa test), **wkh** (the weighted Kishino–Hasegawa test), **wsh** (the weighted Shimodaira–Hasegawa test). The use of wsh is encouraged to minimize Type I error.

Topological hypothesis	au	kh	sh	wkh	wsh
Nephila monophyly	0.096 (−0.006)	0.068 (−0.003)	0.116 (−0.003)	0.068 (−0.003)	0.169 (−0.004)
Nephila + *Nephilengys* + *Herennia*	0.056 (−0.005)	0.043 (−0.002)	0.078 (−0.003)	0.043 (−0.002)	0.103 (−0.003)
Nephilengys monophyly	0.01 (−0.003)	0.006 (−0.001)	0.031 (−0.002)	0.006 (−0.001)	0.015 (−0.001)

contain similarly long branches in some of the conflicting lineages, their dubious phylogenetic position in some of the analyses of the complete matrix may be simply a long branch attraction artifact.

Although the monophyly of nephilids, of araneids containing *Araneus*, *Argiope*, *Caerostris* and others, and of "zygiellids" containing *Zygiella*, *Phonognatha* and *Deliochus*, were well supported in most analyses, relationships between these three major clades remained conflictive and poorly supported.

The topology within Nephilidae is fully resolved and highly similar in all analyses of the molecular matrix (Fig. 2), and the nodes are mostly well supported. All analyses, including combined morphology and molecules, agree that the genera *Herennia* and *Clitaetra* are monophyletic, but that the genera *Nephila* and *Nephilengys* (Kuntner, 2007; Kuntner et al., 2008a) are each diphyletic (Figs. 1 and 2). The true *Nephila* and *Nephilengys* contain species groups that include the type species of each genus, *Nephila pilipes* and *Nephilengys malabarensis* (Walckenaer, 1841), respectively (Fig. 1). The results of the topological tests conducted on the full, concatenated molecular data matrix under alternative topological constraints (Table 2) unequivocally reject the monophyly of *Nephilengys*, but not that of *Nephila* and Nephilinae *sensu* Kuntner et al. (2008a) (all nephilids to the exclusion of *Clitaetra*). Because the monophyly of *Nephilengys* is robustly rejected, we provide a formal reclassification of the species currently under *Nephilengys* (Appendix A). However, since the monophyly of *Nephila* cannot be unequivocally rejected, even though rejected both by combined analyses as well as each gene independently, we here refrain from formally reclassifying those species that might not belong to this genus, but rather discuss those species as belonging to the "*Nephila*" clade.

Results of the divergence time analyses are summarized in Fig. 4. As expected, estimated divergence times are greatly affected by the alternative interpretations of the taxonomic status of the fossil spider *Nephila jurassica*. In the three tested schemes, the origin of extant nephilids was interpreted to have occurred in the mid Jurassic (Fig. 4c), mid-Cretaceous (Fig. 4b) or late Paleocene

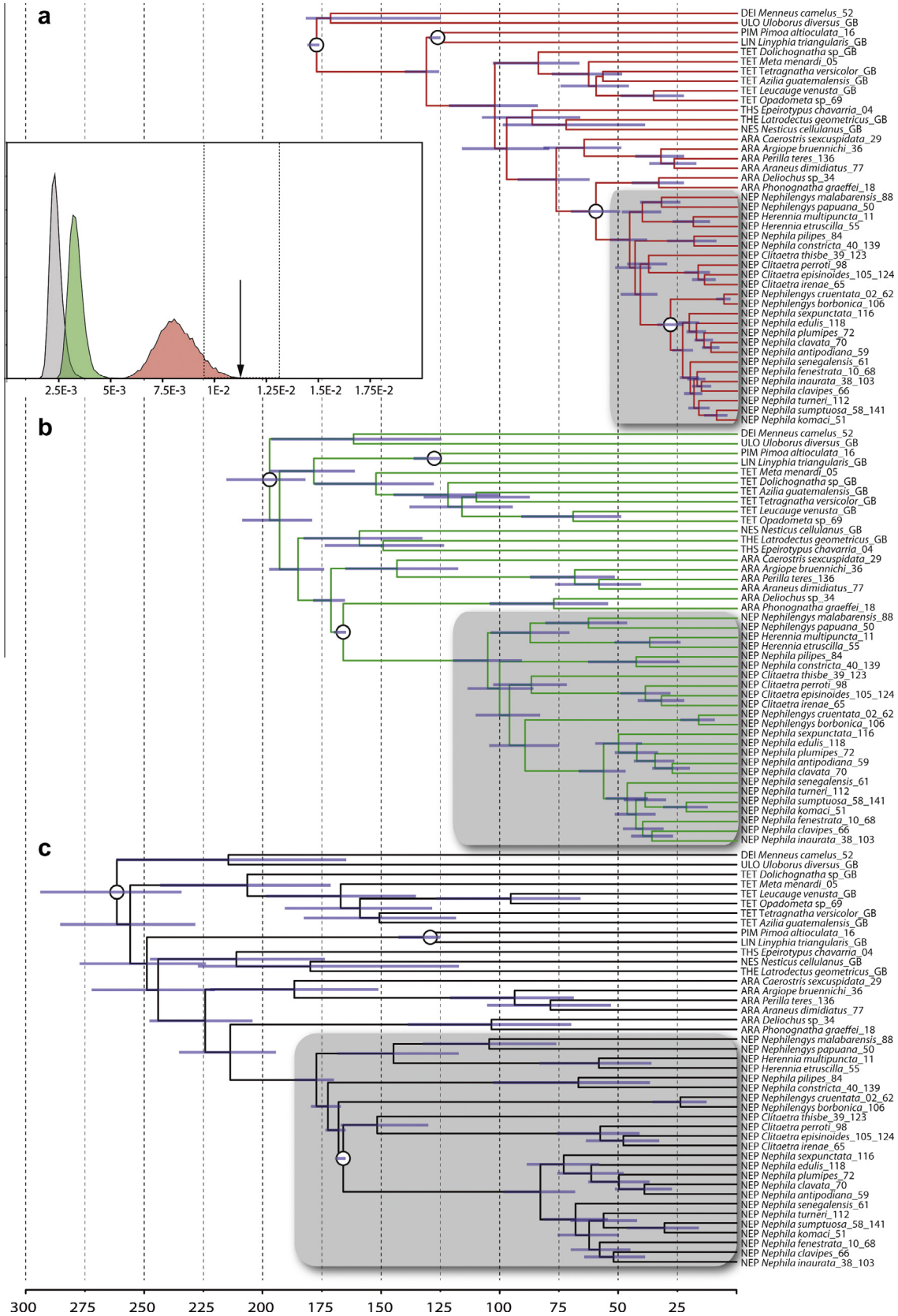

Fig. 4. Chronograms obtained under three different calibration schemes: (a) the fossil *Nephila jurassica* treated as stem orbicularian (red); (b) *N. jurassica* treated as stem nephilid (green); (c) *N. jurassica* treated as stem *Nephila sensu stricto* as implied by the original description (black). Inset plot shows posterior distribution of the ucld.mean parameter for each calibration scheme (color codes as in trees). The arrow and the dotted area in the plot indicate the mean and 95% interval of the ucld.mean estimated by Bidegaray-Batista and Arnedo (2011). Only the scheme shown in a falls roughly within the expected mitochondrial substitution rates. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

(Fig. 4a), according to the different interpretations of the fossil record. The only scheme that infers a substitution rate for the mitochondrial genes partially congruent with the values available in the literature (see Fig. 4 inset) considered *Nephila jurassica* to be neither a *Nephila* nor a nephilid (Fig. 4a). These results suggest that nephilids originated in the late Paleocene to early Eocene, and support the ages of the known Baltic amber fossils as the oldest true nephilids.

4. Discussion

Our results provide a novel interpretation of nephilid phylogeny and robust evidence for its placement and monophyly. Well supported findings also include the monophyly of *Herennia*, the diphyly of *Nephilengys*, and many species level relationships reflecting geographically meaningful groups. Some other findings such as *Nephila* diphyly are repeated in every gene tree, yet fail stringent testing and are thus not as robust. Yet other findings, the monophyly and precise placement of *Clitaetra*, and many outgroup relationships are, however, weakly supported. This discussion highlights these novel findings and their implications for taxonomy, provides evolutionary reinterpretations of key traits, and suggests what the next steps towards obtaining more robust resolution for ambiguous clades might be.

4.1. Nephilid phylogenetic placement, monophyly and exclusivity

Nephilid monophyly has been recovered in all molecular analyses focusing on higher level phylogenetics (Agnarsson et al., 2013; Blackledge et al., 2009; Dimitrov et al., 2012) and those focusing on datasets particular to nephilids (Su et al., 2011) or tetragnathids (Alvarez-Padilla et al., 2009). While these studies mostly only included a small subset of nephilid diversity, typically one *Nephila* and one *Nephilengys* (Agnarsson et al., 2013; Blackledge et al., 2009; but see Dimitrov et al., 2009), or did not include an adequate outgroup selection for such a test (Su et al., 2011), the more complete phylogenetic studies that focused on nephilid morphology and behavior also always confirm nephilids as a well-defined and supported clade (Kuntner, 2005, 2006, 2007; Kuntner and Agnarsson, 2009; Kuntner and Coddington, 2009; Kuntner et al., 2008a). Our results from all analyses also support nephilids as a well-supported clade including 15 unambiguous morphological and behavioral synapomorphies. Among them are the striated cheliceral boss, various sexually dimorphic somatic traits, the presence of the temporary (or auxiliary) spiral in the finished web, and a typical fourth leg laying of the sticky spiral (for details and for other defining features, see Kuntner et al., 2008a). Our results also confirm that *Phonognatha* and *Deliochus* are not nephilids (Kuntner et al., 2008a).

Our results strongly refute the 'tetragnathid hypothesis'placing Nephilinae as a tetragnathid subfamily (Dimitrov and Hormiga, 2009; Griswold et al., 1998; Hormiga et al., 1995; Kuntner and Alvarez-Padilla, 2006). Instead, our results support the monophyly of a clade containing Nephilidae, Araneidae and "Zygiellidae" (all classically araneids). However, the lack of support for deep nodes continues the ambiguity of reconstructed basal araneoid splits (Kuntner, 2005, 2006, 2007; Kuntner and Agnarsson, 2009; Kuntner and Coddington, 2009; Kuntner et al., 2008a). The precise placement of Nephilidae thus remains contentious (Kuntner, 2005, 2006). Beyond the tetragnathid hypothesis, other precisely hypothesized nephilid placements were as sister to araneids (Alvarez-Padilla et al., 2009; Blackledge et al., 2009; Dimitrov et al., 2012), as sister to a clade containing tetragnathids and *Deliochus* + *Phonognatha* (Kuntner, 2006, 2007), or as sister to all other araneoids (Kuntner et al., 2008a), the latter being highly unlikely given our dating results.

Apparently such deep nodes cannot be resolved robustly with the set of molecular markers currently available (Agnarsson et al., 2013). Alternatively, the lack of resolution could be attributed to large amounts of missing data in the outgroups (Table 1), which could even be biasing the resulting phylogeny (Huelsenbeck, 1991). While it is true that missing data may have unpredictable effects on phylogenetic analysis, it has also been shown that positive effects of denser taxonomic sampling usually overwhelm the negative effects of missing data (e.g. Wiens, 2003). In this regard, the largest molecular analysis on orb-web spiders conducted to date (Dimitrov et al., 2012), which included an overall 50% of missing data with some taxa up to 75%, showed improved resolution over prior studies on araneoids that had better gene coverage, yet sparser taxon sampling (Blackledge et al., 2009; Arnedo et al., 2004, 2009). Moreover, the poorly resolved set of outgroups in the current study also comprised taxa with near full gene coverage (e.g. Theridiosmatidae, Theridiidae, Cyatholipidae, Nicodamidae, Synotaxidae). We therefore tend to favor the explanation of Dimitrov et al. (2012) that the lack of resolution in deeper araneoid nodes seems to be not due to missing data but rather due to short branches separating families and fast clock taxa (see Fig. 3).

Our data nevertheless point towards a certain phylogenetic affinity of nephilids with several groups classically belonging to Araneidae. These groups include araneines, argiopines, "zygiellids", *Caerostris* and others though their combined monophyly is only weakly supported in most analyses, and not recovered in others (Fig. 2). The classification implications of our study largely depend on the definition of Araneidae, a huge and poorly defined family containing a hodgepodge of "orb weaving" spiders. The clade, whose diversity surpasses most other spider families, contains well defined lineages such as Araneinae and Argiopinae (Scharff and Coddington, 1997) and other, atypical clades such as "zygiellids" (Gregorič et al., 2010) and *Caerostris* (Kuntner et al., 2008a). Because synapomorphies of such broadly defined Araneidae have been elusive (but, see Scharff and Coddington, 1997), because nephilids continue to be well defined and phylogenetically exclusive from the above and because their precise sister group is ambiguous (araneids *sensu stricto* in Fig. 1 and "zygiellids" in Fig. 2), we support the continued use of the family ranked Nephilidae to include *Nephila, Nephilengys, Herennia,* and *Clitaetra*, and the redefined and renamed species within these classical "genera" (Appendix A; Fig. 5).

Although our phylogeny obviously cannot be taken to resolve the araneid problem, it recovers as a clade typical araneids (*Araneus, Argiope, Cyclosa, Larinioides*), a lineage that is again confirmed by our molecular and combined datasets to also include the Asian *Perilla* (Fig. 2) and the African *Singafrotypa* (Fig. 1) (Kuntner, 2002; Kuntner et al., 2008a; Kuntner and Hormiga, 2002), but, perhaps not the genus *Acusilas* (Fig. 1), although its apparent placement outside araneids might be due to the lack of molecular data. This typical araneid clade is here represented by the terminals from *Caerostris* to *Perilla* (Fig. 2), but is in fact inclusive of dozens other araneid genera (Blackledge et al., 2009; Dimitrov et al., 2012; Scharff and Coddington, 1997). Within this clade we also recovered the Australian leaf-curling spider *"Araneus" dimidiatus* (Fig. 2), but since it does not group with true *Araneus* it should be classified in a different genus. As stated above, our topologies from different analyses and datasets do not resolve the relationships among "zygiellids", araneids and nephilids (Figs. 1 and 2), reflecting the lack of consensus also among the results from other independent molecular analyses (Blackledge et al., 2009; Dimitrov et al., 2012). Clearly, these relationships and precise composition of Araneidae versus "Zygiellidae" will remain a topic for future research, with the most likely significant progress coming through novel genomic approaches (Agnarsson et al., 2013).

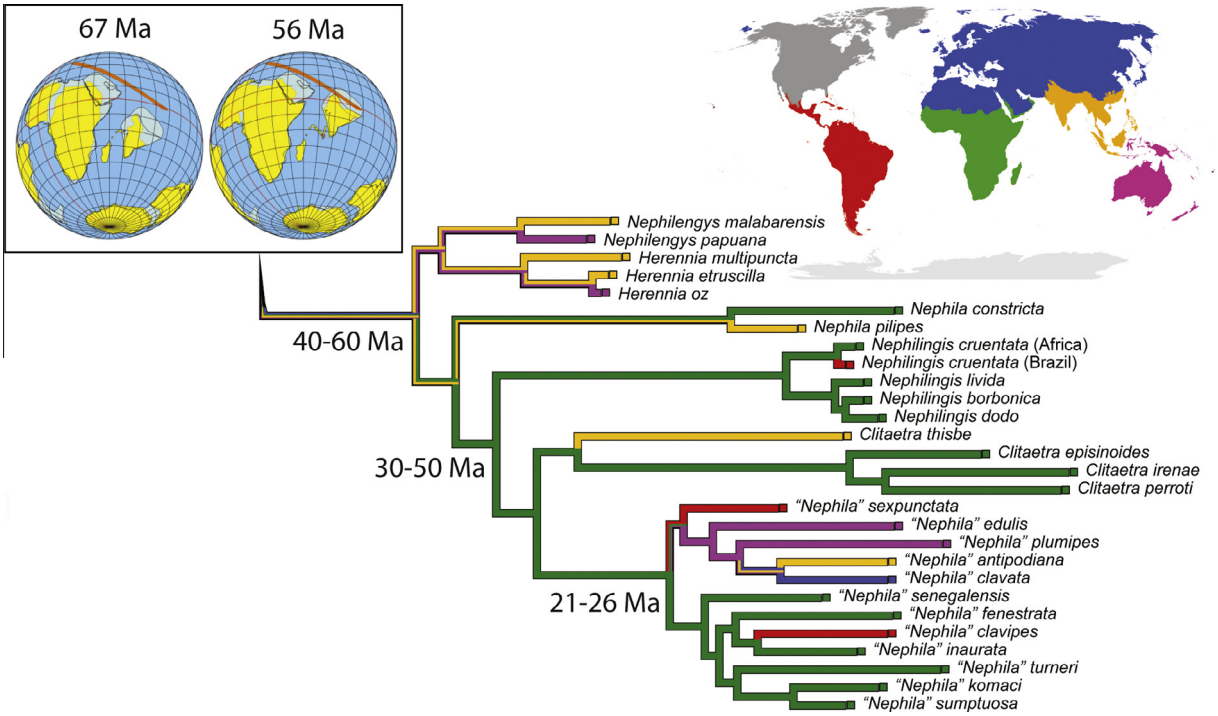

Fig. 5. A summary nephilid phylogeny based on the Bayesian tree in Fig. 2 with squares at terminals color coded according to biogeographical regions (see right map inset). Branches are also color coded for geography, with the ancestral values inferred using parsimony optimization. Although the tree is not ultrametric (all terminals are in fact contemporary) the roughly estimated main clade ages are labeled according to the scheme A in Fig. 4. The nephilid ancestral age is thus between 40 and 60 million years when the Gondwanan continents were already largely split (see left map inset).

4.2. Phylogenetic structure within Nephilidae

Kuntner et al. (2008a) recovered two sister nephilid clades. Nephilinae contained the typical representatives of the family with large females: *Nephila*, *Nephilengys* and *Herennia;* Clitaetrinae included only *Clitaetra*, a genus of poorly known small spiders of African and Indian tropics. Each of the four genera was monophyletic with well-defined morphological and behavioral synapomorphies. *Clitaetra* and *Herennia* had representatives with specialized arboricolous ladder webs (Kuntner, 2005, 2006; Kuntner and Agnarsson, 2009; Kuntner et al., 2008b), *Nephilengys* had larger spiders building tree-trunk webs with retreats (Kuntner, 2007; Kuntner and Agnarsson, 2011a; Kuntner et al., 2010b), and *Nephila* contained the largest, long-legged nephilids, all of which build aerial webs with golden threads and with three dimensional barrier webs (Kuntner et al., 2008a, 2010a,b). *Nephila* was sister to *Nephilengys* (Kuntner et al., 2008a), competing with the alternative hypothesis stating that *Nephila* was sister to a clade *Herennia* + *Nephilengys* (Alvarez-Padilla et al., 2009; Dimitrov et al., 2009, 2012; Dimitrov and Hormiga, 2009; Hormiga et al., 1995). Our results, not sensitive to any analytical approach, only confirm the monophyly of *Herennia* and *Clitaetra*, but not of the Nephilinae. Most surprisingly, the two classical nephilid genera, *Nephila* and *Nephilengys* are also not recovered monophyletic in any analysis. However, while our approximation unbiased test flatly rejected *Nephilengys* monophyly, the monophyly of *Nephila* and Nephilinae could not be rejected.

4.2.1. Nephila diphyly

Nephila monophyly has never before been questioned, and the genus was well supported by morphological phylogenetics (Kuntner and Coddington, 2009; Kuntner et al., 2008a). It is therefore surprising that the defining features of *Nephila*, such as aerial web, golden silk, and the typical male palpal conformation, should all collapse as synapomorphies at this level. Although the topology tests caution against rejecting *Nephila* monophyly, our unconstrained analyses, agreement among independent gene partitions, and combined molecular and morphological data, suggest the opposite (Figs. 1 and 2). Among species known as *Nephila* (Kuntner and Coddington, 2009; Kuntner et al., 2008a), the Asian *N. pilipes* groups with the African *N. constricta*, a sister relationship also supported by previous morphological (Kuntner et al., 2008a) and molecular studies (Su et al., 2011). As *N. pilipes* is the type species of the genus *Nephila*, this clade will continue to bear the name *Nephila*. Because the other "*Nephila*" clade, which contains the remainder of the classical *Nephila* species, groups with *Clitaetra* (Fig. 2) or with *Clitaetra* + *Nephilingis* (Fig. 1), currently *Nephila* species other than *N. pilipes* and *N. constricta* should be formally transferred to another genus. Being that golden orb-weavers are models for much ongoing research, such a nomenclatural act would likely frustrate many researchers and should preferably be made if and when *Nephila* monophyly is unambiguously rejected; we therefore refrain from doing so here. This large "*Nephila*" clade has two subclades, one with mostly Australasian species including the Eurasian *N. clavata* L. Koch, 1878 but also the American *N. sexpunctata*, and the other with all African giants (Kuntner and Coddington, 2009) but also with the American *N. clavipes* (Linnaeus, 1767).

4.2.2. Nephilengys diphyly

The two species groups within *Nephilengys sensu* Kuntner (2007), the *cruentata* and the *malabarensis* species groups are in fact distinct clades emerging at different parts of the phylogeny, as already hinted by the population level analyses of the Indian Ocean *Nephilengys* (Kuntner and Agnarsson, 2011a). All our results show that classical *Nephilengys* is diphyletic and the approximation unbiased test also rejects *Nephilengys* monophyly. The clade containing *Nephilengys cruentata, N. livida, N. borbonica,* and *N. dodo*, is here transferred to a new genus, *Nephilingis* Kuntner (Appendix A). Meanwhile, the Australasian clade continues to be classified

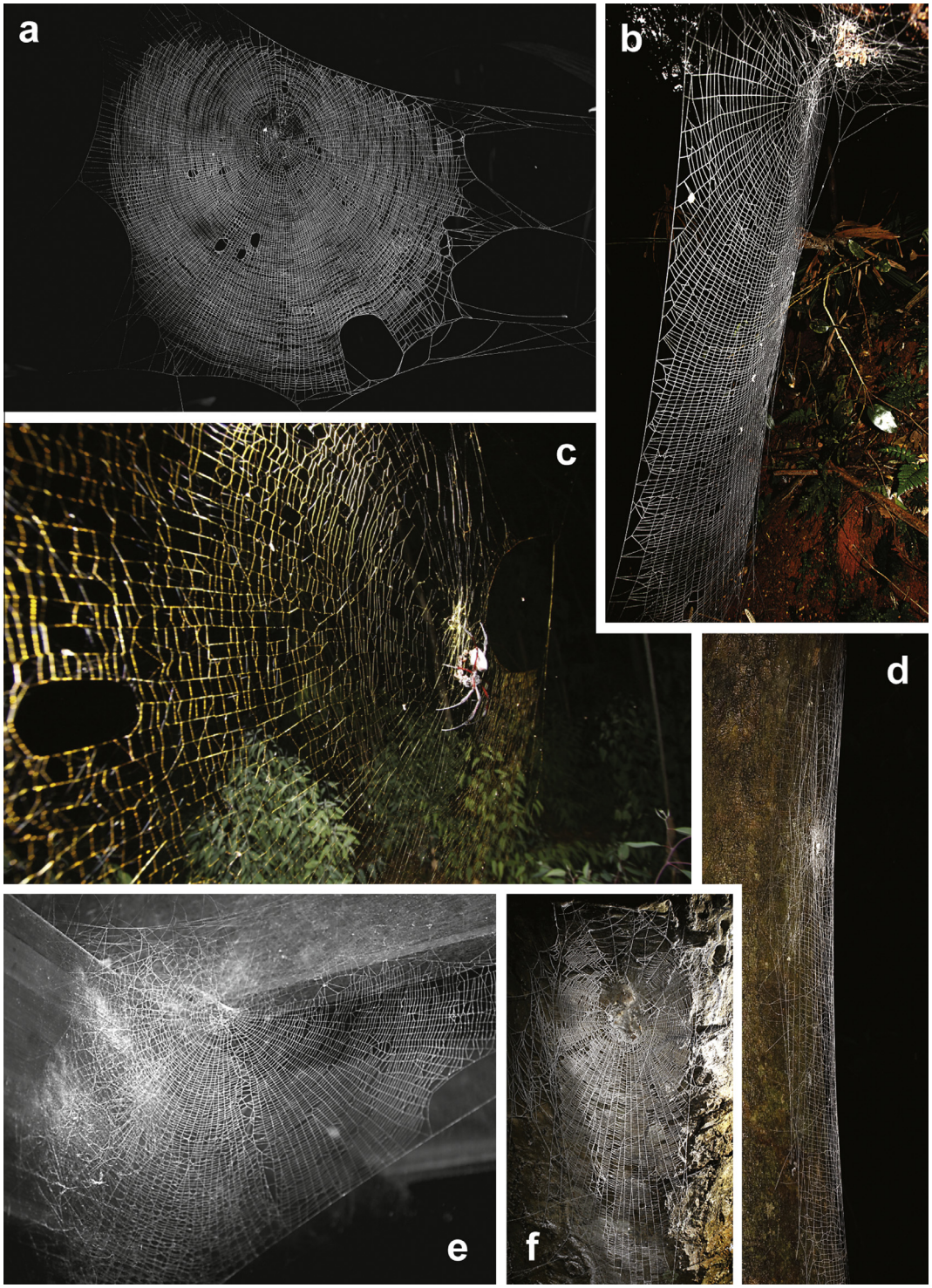

Fig. 6. Typical web architectures of the six nephilid genera mirroring the phylogenetic results: (a) *Nephila* (*N. pilipes*); (b) *Nephilingis* (*N.* n. sp. from Seychelles); (c) "*Nephila*" (*N. inaurata*); (d) *Herennia* (*H. multipuncta*); (e) *Nephilengys* (*N. papuana*); (f) *Clitaetra* (*C. episinoides*).

in *Nephilengys* because the type of the genus is *N. malabarensis*. *Nephilengys* is now recovered as sister to *Herennia* (Hormiga et al., 1995), also Australasian in origin. Since *Nephilingis* is either sister to *Clitaetra* (Fig. 1) or to *Clitaetra* + "*Nephila*" (Fig. 2), the previously inferred sister group relationship *Nephila* + *Nephilengys* (Kuntner et al., 2008a) is rejected. Prior studies, of course, all falsely assumed the monophyly of Afrotropical and Autralasian "*Nephilengys*" species (Alvarez-Padilla et al., 2009; Hormiga et al., 1995; Kuntner, 2007; Kuntner et al., 2008a). Our data suggest that several aspects of biology of these two clades, previously interpreted as synapomorphies for the genus, are in fact plesiomorphic, perhaps even ancestral to nephilids. These features include the web architecture, which is in both lineages a large asymmetric orb built against trees or other substrate, and always possessing a retreat (Kuntner, 2007; Kuntner and Agnarsson, 2011a; Kuntner et al., 2010b), and the habit of males to sever and lose their palps during or after mating, which renders them sterile eunuchs (Kuntner, 2007). The non-monophyly of *Nephilengys* can also explain the subtle differences in the mechanisms and behavioral outcomes of palpal mutilation and emasculation between *Nephilengys*

malabarensis and *Nephilingis livida* (Kralj-Fišer et al., 2011; Kralj-Fišer and Kuntner, 2012).

4.3. Nephilid cladogenetic and biogeographic history

Two of the tree calibration schemes tested, the one that interpreted the fossil *Nephila jurassica* to represent a true *Nephila* dating from the Jurassic and the one that interpreted it as a stem nephilid, were discarded in favor of the third scheme interpreting it as a stem orbicularian. This conclusion, based on the comparison of calibrated phylogenies and the expected mitochondrial substitution rates (Fig. 4; ESM D), plus our interpretation of the morphology of the fossil, strongly suggests that *N. jurassica* is not a nephilid, much less a *Nephila*, and that its classification (Selden et al., 2011) was most likely based on erroneous interpretations of its morphological features. The placement of this cribellate fossil as a stem orbicularian is thus more consistent with its morphology, and age. These results imply that *Nephila* does not date back to the Jurassic, but rather represents a much more recent lineage (see below). The 40 Ma old *Palaeonephila* from Baltic amber is thus the oldest fossil we can with some certainty confirm to belong to Nephilidae.

We here provide a preliminary reinterpretation of historic biogeographic events in nephilids based on a simple parsimony model of optimized areas on the Bayesian tree (Fig. 5) and on the clade ages inferred under scheme in Fig. 4a. Given an estimated nephilid ancestral age between 40 and 60 million years when the Gondwanan continents were already largely split (see inset in Fig. 5), it is unlikely that the family is Gondwanan and vicariant (*contra* Kuntner, 2006). Instead, the nephilid ancestor had to originate on a more or less formed contemporary continent. The origin of the family is in the Old World tropics, but further detail is ambiguous, with the origin equally likely to be Africa, Asia, or Australasia (Fig. 5). The most basal and well supported clade within Nephilidae, *Herennia* plus *Nephilengys*, contains only Australasian species. The ancestral area optimization at the next node is also ambiguous, but the next distal node containing all other nephilids (not the doublet of true *Nephila*) is already unambiguously African, containing a clade of Afrotropical representatives (*Nephilingis*), wherein one very recent dispersal event to the neotropics is postulated (*N. cruentata*), *Clitaetra* wherein one dispersal event is postulated over the Indian Ocean to Sri Lanka, and the clade of other "*Nephila*", also of African origin. The latter contains two sister groups, one with an African origin and one dispersal to the neotropics (*N. clavipes*), and the other of ambiguous origin and with likely dispersals to the neotropics (*N. sexpunctata*), to Australasia (ancestor to all others), and more distally with postulated dispersal(s) to tropical (*N. antipodiana* Walckenaer, 1841) and temperate Asia (*N. clavata*).

As always, more data are needed for a better resolution and more definitive biogeographic interpretations, however, these initial interpretations do not contradict those of Su et al. (2011) who hypothesized either an African or Asian origin of *Nephila* s.l. (including *N. pilipes* and *N. constricta* as well as "*Nephila*" species), and established the main cladogenetic events within *Nephila* s.l. to have been in mid-Miocene to Pliocene some 16–2 million years ago. Likewise, our results are congruent with those suggesting a recent diversification in the Indian Ocean *Nephilingis* lineages (Kuntner and Agnarsson, 2011a) and *Nephila inaurata* populations (Kuntner and Agnarsson, 2011b).

4.4. Evolution of size and sexual size dimorphism

Previous nephilid phylogenetic topology, i.e. *Clitaetra* + Nephilinae (Kuntner et al., 2008a), suggested a small hypothesized ancestral female size, a monotonic increase of female body size throughout nephilid evolution, and a decoupled evolution of male size, the latter showing no clear trends in increase or decrease (Kuntner and Coddington, 2009). Clearly, the conclusions of Kuntner and Coddington are challenged by our new phylogeny, although of course, our reinterpretations are preliminary due to the ambiguous rejection of *Clitaetra* + Nephilinae relationship and the ambiguous *Nephila* diphyly. Nevertheless, parsimony would suggest that a shift to female gigantism happened much more ancestrally than hypothesized in the above study. Giant size might in fact optimize at least to the node subtending *Nephila, "Nephila", Nephilingis* and *Clitaetra*, with a reversal to small size in the ancestor to *Clitaetra*. Female size of ancestral nephilids was therefore medium, not small, and gigantism occurred early in the evolution, and repeatedly, and not only distally as hypothesized by Kuntner and Coddington (2009). The picture is likely to change, and strongly depends on a better reconstruction of the immediate nephilid outgroup, and of deep nodes. One should add that phylogenetic patterns alone are not sufficient to explain the evolution of nephilid sexual size dimorphism. Recent studies have established the importance of phenotypic plasticity in nephilids. For example, a genotype can produce a huge size variation under varying ecological conditions (Higgins et al., 2011), and post-maturity female molting can facilitate female gigantism in some but not all individuals, depending on female mating history (Kuntner et al., 2012a,b).

4.5. Evolution of sexually antagonistic traits

Nephilids show an amazing variety of mating behaviors and strategies, ranging from extreme monogamy to extreme polygamy, where male mating plugs are either fully effective or completely ineffective; this diversity within a small lineage has been hypothesized to arise through processes of sexually antagonistic coevolution (Kuntner et al., 2009b). *Herennia*, *Nephilengys*, and *Nephilingis* males perform plugging via genital mutilation (Kuntner, 2005, 2007; Kuntner et al., 2009a,c) and females exhibit correspondingly high levels of sexual cannibalism (Kralj-Fišer et al., 2011, 2012; Kralj-Fišer and Kuntner, 2012), hinting at sexual conflict. *Clitaetra* is not known for genital plugs and their mating biology is virtually unknown, but in *Nephila pilipes* and *N. constricta*, extreme female polyandry has been documented with correspondingly ineffective male-mutilated plugs (Kuntner et al., 2008a, 2009b,c). Furthermore, *N. pilipes* males perform mate binding, a ritual that calms females to reduce their aggression and increase receptivity (Zhang et al., 2011), and females produce their own plugs, thereby sealing off their genitals to prevent excessive copulations (Kuntner et al., 2012a). Cases of both monogamy and polygamy are known in other "*Nephila*" species (Fromhage et al., 2007; Schneider et al., 2001, 2005), where males evolved one-shot genitalia (Christenson, 1989; Michalik and Rittschof, 2011; Schneider and Michalik, 2011). Reinterpreting the evolution of sexually antagonistic traits on the species level phylogeny is preliminary, but it seems that male enforced monogamy is ancestral in the family, and is retained in *Nephilengys*, *Herennia*, and in *Nephilingis*, where the males, all possessing complex sexual organs, could be interpreted to have the upper hand in sexual control (Kuntner et al., 2009b). Rather than a single shift in simplification of male and female genitals with a corresponding shift towards polygamy (Kuntner et al., 2009b), it seems that such a concerted shift must have happened at least twice, once in each "*Nephila*" clade. Being that *"Nephila" fenestrata* is now recovered much more distally, a further shift back to functional plugs and male enforced monogamy now seems plausible, further complicating the arms race scenario (Kuntner et al., 2009b).

4.6. Web evolution

Intuitively, it may be difficult to understand the collapse of the golden webs as *Nephila* synapomorphy (Fig. 6c), as a strictly

parsimonious interpretation of the character distribution on the new phylogeny would suggest that this trait arose twice (or that it was lost twice). Craig et al. (1996) demonstrated that *Nephila clavipes* can produce silk of varying spectral properties in response to the intensity and spectral composition of its light environment. In bright light, *N. clavipes* produced a complex of yellow pigments that absorb light of wavelengths less than 500 nm, making their webs appear golden. In dim light and at wavelengths less than 500 nm the spiders produced pigments that reflect light between 400 and 500 nm, making their webs appear white. Hence, the ability to produce webs of golden color, depending on the microhabitat, may be present in a wider clade than just the traditional "*Nephila*", but the golden shine only shows in those spiders spinning aerial webs in bright light, corresponding to the two clades traditionally named *Nephila* (Fig. 6a and c). Furthermore, Craig et al. (1996) also found that yellow silks may attract herbivorous and pollinating insect prey to spider webs. Perhaps the expression of golden silk production is subject to more ecological than phylogenetic factors.

Within Nephilidae, it has been hypothesized that aerial webs arose from substrate webs once in the ancestor to *Nephila* (Kuntner et al., 2008a, 2010a,b). Although we could not unequivocally reject *Nephila* monophyly, Figs. 1 and 2 suggest either two independent origins of true "*Nephila*" aerial webs (Fig. 2), or a single origin, but with a reversal to tree webs in the common ancestor to *Nephilingis* and *Clitaetra* (Fig. 1; Fig. 6b and f). Although ambiguous, the following interpretation of nephilid web evolution seems plausible.

The plesiomorphic condition in Nephilidae is a partially arboricolous, asymmetric orb web with a retreat, as exemplified by both groups of extant "*Nephilengys*" (Fig. 6b and e) (Kuntner et al., 2010b). Such architecture, utilizing the tree trunk and a major branch as substrate/roof, having most of the planar orb aerially exposed, and sporting a retreat with the daytime hiding spider in the dry eave, was retained by both "*Nephilengys*" clades, and is thus symplesiomorphic. This architecture was modified into true arboricolous webs independently in *Herennia* (Fig. 6d) and in *Clitaetra* (Fig. 6f).The *Herennia* web is an elongate, specialized arboricolous structure closely adhering to tree trunk curvature through the apomorphic use of pseudoradii (Kuntner, 2005; Kuntner et al., 2010b). *Clitaetra* webs are also truly arboricolous, but are less elongate and are moved centimeters away from the trunk, thereby retaining planarity (Kuntner, 2006; Kuntner and Agnarsson, 2009; Kuntner et al., 2008b). From the architectural, functional, and size perspectives, the independent evolution of these two tree webs seems logical. Finally, the topology in Fig. 2 suggests that the plesiomorphic, partially arboricolous and asymmetric orb web became aerial twice in the ancestors to both "*Nephila*" groups. Becoming fully aerial means freeing itself from the constraints of substrate (tree trunk) to become larger, and thus the lost retreat is a logical consequence. Literature suggests a link between females becoming free from substrate and being able to better respond to fecundity selection (Kuntner and Coddington, 2009). What ensues is the evolution of female body- and web-gigantism (Kuntner et al., 2010a,b, 2012b). From our results it seems that such bursts of size evolution happened twice, in both cases leading to "*Nephila*" clades with females of extreme sizes, reaching independent pinnacles of female gigantism in *Nephila pilipes* + *constricta*, and perhaps more gradually, in the "*Nephila*" clade.

5. Conclusions

Our study provides the first species level nephilid molecular phylogeny as a test of their relationships and evolutionary history independent of much debated and controversial morphological and behavioral datasets. Although disputing the Jurassic *Nephila* fossil record, we showed that this 40–60 million year old lineage of giant orb-weaving spiders has had a long, independent evolutionary history (Fig. 5). The biogeography and evolution of nephilids can now be reinterpreted based on the new phylogeny, which also provides a basis for a natural classification, though further data are necessary to settle outstanding issues. Although plausible, our interpretations need to be taken as preliminary in the light of poorly supported deep nodes, and the ambiguity regarding the monophyly of *Nephila* and Nephilinae. Precise details of the nephilid topology will no doubt change further as more data become available. Nevertheless, we believe that this new phylogeny represents substantial progress and will facilitate new research of this model lineage, which has already been prominent in ecological, behavioral, morphological and physiological studies.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ympev.2013.06.008.

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