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#### Running title: Phylogeny and biogeography of Pachygoneae

# Phylogeny and biogeography of Pachygoneae (Menispermaceae), with consideration of the boreotropical flora hypothesis and resurrection of the genera *Cebatha* and *Nephroia*

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#### ABSTRACT

The tribe Pachygoneae consists of four genera with about 40 species, primarily distributed in tropical and subtropical Asia and America, also in Australasia and Africa. This tribe presents an ideal model to investigate the origin of the tropical and subtropical amphi-Pacific disjunction pattern. More specifically, it allows us to test whether the tropical lineages diverged earlier than the subtropical ones during the fragmentation of the boreotropical flora. In this study, we reconstructed the phylogeny of Pachygoneae using five plastid (*rbcL*, *atpB*, *matK*, *ndhF*, *trnL*-F) and one nuclear (26S rDNA) DNA regions. Our results indicate that Pachygoneae is not monophyletic unless Cocculus pendulus and Cocculus balfourii are excluded. We resurrected the genus *Cebatha* to include these two species and established a new tribe for this genus. Within Pachygoneae, the species of *Cocculus* are distributed in three different clades, among which two are recognized as two distinct genera, Cocculus s.str. and Nephroia resurrected, and one species is transferred into Pachygone. Our molecular dating and ancestral area reconstruction analyses suggest that Pachygoneae began to diversify in tropical Asia around the early-middle Eocene boundary (c. 48 Ma) and expanded into the New World by c. 44 Ma. In the New World, tropical Hyperbaena originated in the late Eocene (c. 40 Ma), whereas the subtropical *Cocculus carolinus* and *Cocculus diversifolius* originated later, in the early Oligocene (c. 32 Ma). These two timings correspond with the two climatic cooling intervals, which suggests that the formation and breakup of the boreotropical floral may have been responsible for the

amphi-Pacific disjunct distribution within Pachygoneae. One overland migration event from Asia into Australasia appears to have occurred in the early to late Miocene.

*Keywords:* Amphi-Pacific disjunction; Biogeography; Boreotropics; *Cocculus*; Menispermaceae; Phylogeny

#### 1. Introduction

Understanding how intercontinental disjunct distributions of organisms have been shaped is a fundamental question in biogeography (Raven, 1972; Lomolino et al., 2010). The amphi-Pacific tropical disjunction has fascinated biogeographers and palaeoecologists since van Steenis and Thorne (van Steenis, 1962; Thorne, 1972). Taxa with such a disjunction are found in tropical and subtropical regions on both sides of the Pacific Ocean with the highest biodiversity on Earth (Wu, 1983; Raven, 1988; Van Damme and Sinev, 2013). In plants, the disjunct distributions between eastern Asia and North America have been extensively studied in temperate elements (Donoghue and Smith, 2004; Wen et al., 2010, 2016). Few studies have been devoted to investigate the amphi-Pacific tropical disjunction (Li and Wen, 2013; Huang et al., 2016).

Thorne (1972) listed 89 genera, 4 tribes or subtribes, 3 or 4 subfamilies, and 8 to 11 families of angiosperms that exhibit the amphi-Pacific tropical distribution. How these taxa achieved their present-day amphi-Pacific disjunct ranges is still poorly understood. Wolfe (1975) proposed the "boreotropical flora" hypothesis to explain this pattern. This hypothesis postulates that during climatically warm periods of the Paleogene, the boreotropical flora with many tropical and subtropical elements spanned the mid-latitudes of the Northern Hemisphere and populations could exchange between areas via land bridges, then owing to the climatic deterioration during the late Eocene, boreotropical elements migrated southwards following the shrinking of subtropical and tropical zones of Asia and America, finally resulting in the amphi-Pacific tropical disjunction pattern (Wolfe 1975; Tiffney 1985a). When the estimated ages for the splits of the tropical lineages in both continents are during the middle to late Eocene, the boreotropical flora hypothesis is usually adopted as the most likely explanation (e.g., Nie et al., 2008; Li et al., 2011; Li and Wen, 2013; Huang et al., 2016; Liu et al., 2016; Yang et al., 2018). Based on this hypothesis, as the period of climate cooling in the late Eocene may have caused the thermophilic lineages to move to lower latitudes, the tropical lineages should have separated earlier than the subtropical and temperate. However, this scenario has never been tested in a phylogenetic framework by including closely related elements spanning the tropical and subtropical zones.

The tribe Pachygoneae (Menispermaceae) was re-delimited by Ortiz et al. (2016) based on molecular and morphological data. This re-delimited tribe comprises four genera and about 40 species (Fig. 1; Ortiz et al., 2016). *Haematocarpus*, containing 2 species, is endemic to Southeast Asia. *Pachygone*, consisting of 12 species, is primarily distributed in Southeast Asia, as well as in northeastern Australia and adjacent islands. *Hyperbaena* contains about 22 species, which are entirely restricted to the tropical America. *Cocculus* currently contains about 8 species, among which three species are found in tropical and subtropical Asia and eastern Asia, two in subtropical North America, one in tropical Africa, and two from tropical Asia to Africa (note that *Cocculus madagascariensis*, apparently from Madagascar was based

on a mislabeled collection that was actually from Central America, likely from Mexico; Forman, 1974a). Thus, the distribution patterns of this tribe provide an opportunity to test the boreotropical flora hypothesis.

Of the four genera in Pachygoneae, only the circumscription of *Cocculus* has been controversial. Based on the plastid (*mat*K and *trn*L-F) and nuclear (ITS) sequences, Wang et al. (2007) found that *Pachygone* was nested in *Cocculus*. In the same year, Ortiz et al. (2007) analyzed the plastid *ndh*F data and also found a non-monophyletic *Cocculus*, in which the sampled four species of the genus were distributed in three clades. Similar results were found in subsequent studies (Hoot et al., 2009; Jacques et al., 2011; Wang et al., 2012; Ortiz et al., 2016), although Wefferling et al. (2013) found that Bayesian inference (BI) supported *Cocculus* as monophyletic, whereas maximum parsimony (MP) and maximum likelihood (ML) analyses recognized it to be polyphyletic. No more than four species of the genus were included in each of the aforementioned phylogenetic analyses. Importantly, the type species of *Cocculus*, *C. hirsutus*, has not been sampled in any molecular study, which has prevented us from updating the classification of this genus.

In this study, our objectives are (1) to reconstruct phylogenetic relationships within Pachygoneae using six plastid and nuclear DNA regions with a focus on *Cocculus*, hence with a more comprehensive sampling than in any previous study, (2) to clarify the circumscription of *Cocculus*, and (3) to investigate the historical biogeography of the tribe in the context of the boreotropical flora hypothesis.

#### 2. Materials and methods

#### 2.1. Taxon sampling and DNA sequencing

We sampled 35 taxa, representing all four of the currently recognized genera of Pachygoneae (Fig. 1; Table S1). Our sampling encompasses all eight species of *Cocculus*, among which its type species, *C. hirsutus*, was included in a molecular phylogenetic analysis for the first time. Thirty additional species were sampled as outgroups, representing all other six tribes in the Menispermoideae (Ortiz et al., 2016). Menispermeae were used to root the tree based on the results of Wang et al. (2012) and Ortiz et al. (2016).

Six DNA regions, including plastid (*rbcL*, *atp*B, *mat*K, *ndh*F, *trn*L-F) and nuclear (26S rDNA) loci, were used in this study. Genomic DNA extraction, amplification, sequencing, and alignments followed Wang et al. (2012, 2016). Voucher information and GenBank accession numbers are listed in Table S1.

#### 2.2. Phylogenetic analyses

Phylogenetic analyses of the cpDNA (*rbcL*, *atp*B, *mat*K, *ndh*F, *trn*L-F), 26S rDNA and combined cpDNA and 26S rDNA datasets were conducted using ML and BI methods in RAxML 8.0 (Stamatakis, 2014) and MrBayes 3.2.5 (Ronquist et al., 2012), respectively. RAxML was performed with the GTR +  $\Gamma$  substitution model for each DNA region, and nodal support was assessed using the fast bootstrap option with 1000 replicates. For BI analyses, each DNA region was assigned its own model of nucleotide substitution, as determined by the Akaike Information Criterion (AIC) in jModeltest 2.1.4 (Posada, 2008). Two independent runs, each comprising four Markov Chain Monte Carlo (MCMC) chains, were conducted, sampling one tree every 1000 generations for 50 million generations, starting with a random tree. Stationarity was determined in Tracer 1.6 (Rambaut et al., 2014). Majority rule (> 50%) consensus trees were constructed after removing the burn-in period samples (the first 25% of sampled trees). Data homogeneity between the cpDNA and 26S rDNA was examined using the incongruence length difference (ILD) test (Farris et al., 1995), implemented in PAUP\* 4.0b10 (Swofford, 2003). This test employed 1000 replicates, each with 10 random additions using tree-bisection-reconnection branch swapping.

#### 2.3. Divergence time estimation

Divergence times were estimated using the combined six locus data in BEAST 2.1.3 (Drummond et al., 2012). We used a reduced dataset of 50 taxa of Menispermoideae that kept one accession per species. Phylogenetic analyses (Ortiz et al., 2007, 2016; Wang et al., 2012; this study) indicate that the species of *Cocculus* are distributed in four clades. Thus, we did not use *Cocculus lottii* from the early Middle Eocene of Germany (Collinson et al., 2012) as a calibration point. Here we selected six fossils that can be confidently placed in our tree (see Table S2 for the detailed justifications of their placements). Following the suggestion of Parham et al. (2012),

we used the uniform prior distribution for these six calibrating fossils. The maximum age of the root was set at 100 Ma, which is the estimated crown age of the Menispermoideae (Wang et al., 2012), with a normal distribution and standard deviation of three.

We first used Bayes factors (Kass and Raftery, 1995) calculated by marginal likelihoods derived from path sampling (Baele et al., 2013) to test four clock models (strict, exponential, lognormal, and random), and then to test two distinct tree speciation priors (Yule and birth-death, BD). Under the best-fit clock model and tree prior, the BEAST analysis was run relied on the GTR + I +  $\Gamma$  model for each DNA partition separately. The MCMC chains were run for 50 million generations, sampling every 5000 generations. Convergence and the adequate effective sample size values (> 200) were checked in Tracer 1.6 (Rambaut et al., 2014). The maximum clade credibility (MCC) tree with median branch lengths and 95% highest posterior density (HPD) intervals on nodes was then built using TreeAnnotator 2.1.3 (part of the BEAST package) with the initial 25% of trees discarded as burn-in. For comparative purpose, we also estimated divergence times using the six-locus dataset with all samplings (65 taxa). This analysis was conducted as described above.

#### 2.4. Biogeographical analyses

Ree and Sanmartín (2018) suggested that the dispersal-extinction-cladogenesis (DEC) model with + J parameter is invalid because of errors in estimation of

likelihoods. Here ancestral areas at internal nodes within the phylogenetic tree were inferred using the DEC model, implemented in Lagrange 2.0.1 (Ree and Smith, 2008). The species-level MCC tree generated from the BEAST analysis was used as the input tree. Four areas of endemism were defined according to the distribution of the Pachygoneae: (A) tropical and subtropical Asia, (B) tropical and subtropical America, (C) Africa, and (D) Australasia. Referring to Mao et al. (2012), we specified dispersal probabilities between pairs of areas for four separate time slices. The maximum number of areas in the ancestral ranges was constrained to two, as no extant species of Pachygoneae is distributed in more than two areas of endemism.

#### 3. Results

#### 3.1. Phylogenetic analyses

The aligned cpDNA dataset included 7244 characters: *rbc*L, 1385 bp; *atp*B, 1406 bp; *mat*K, 1250 bp; *ndh*F, 2060 bp; and *trn*L-F, 1143 bp. The aligned 26S rDNA sequences were 1328 bp nucleotides in length. The ILD test value was P = 0.34, indicating that the cpDNA and 26S rDNA datasets are not significantly different from random partitions of the combined dataset. Except for the presence of several nodes with weak support (BS < 70% and PP < 0.95), the topology of Menispermoideae phylogeny generated based on the cpDNA dataset (Fig. S1) was highly congruent with the 26S rDNA tree (Fig. S2).

The combined cpDNA and 26S rDNA dataset comprised 8572 characters. ML

and BI analyses resulted in identical trees with strong support for most nodes (Fig. 2). *Cocculus pendulus* and *C. balfourii* formed a clade (*Cocculus* 1; BS = 80%, PP = 0.99), which is in an unresolved position in relation to Pachygoneae, Tiliacoreae, Spirospermeae, and Cissampelideae. Within Pachygoneae, *Haematocarpus* is sister to the remaining taxa of the tribe (BS = 66%, PP = 0.94), followed by *Cocculus hirsutus* (*Cocculus* 2; BS = 87%, PP = 1.0), then *Hyperbaena* (BS = 86%, PP = 0.95). *Hyperbaena* is strongly supported as monophyletic (BS = 99%, PP = 1.0). *Cocculus* 3 clade contains *Cocculus carolinus*, *C. diversifolius*, and *C. orbiculatus* (including *C. trilobus* Mayuzumi 3826 and *C. trilobus* Tomoko Fukuda *s.n.*) (BS = 93%, PP = 1.0). *Cocculus laurifolius* (*Cocculus* 4) is embedded within *Pachygone* and is sister to *P. valida* (BS = 92%, PP = 1.0). Similarly, *C. trilobus* Hong YP H310 is nested in *Pachygone* and forms a clade with *P. sinica* (BS = 100%, PP = 1.0).

#### 3.2. Divergence time estimation and biogeographical reconstruction

For the 50-taxon and 60-taxon data, the lognormal clock model and BD tree prior were identified as best-fit (Tables S3 and S4). Divergence time estimates based on the two different sampling strategies are highly congruent (Figs. 3 and S3). The species-level age estimation is here reported and was used for biogeographical analysis. Ancestral area inference is indicated in Figs. 4 and S4 and Table S5. The five Menispermoideae lineages (Pachygoneae, *Cocculus* 1-i.e., Cebatheae, Tiliacoreae, Spirospermeae, and Cissampelideae) started to diverge rapidly at 72.69 Ma (95% HPD: 63.88–83.05; node 1). *Cocculus* 1 diverged from Pachygoneae at 66.92 Ma (95% HPD: 54.43–79.59; node 2). The most recent common ancestor of Pachygoneae was likely present in tropical Asia at 47.65 Ma (95% HPD: 33.33–61.44; node 3). One dispersal event was inferred from Asia to the New World (between nodes 3 and 4). The age of node 4 was estimated at 44 Ma (95% HPD: 33.46–56.74). Two American lineages, *Hyperbaena* and *C. carolinus-C. diversifolius*, originated at 40.07 Ma (95% HPD: 30.20–52.08; node 5) and 32.49 Ma (95% HPD: 20.80–44.63; node 6), respectively. The dispersal from Asia into Australia took place at *c*. 20.24–8.11 Ma (between nodes 7 and 8).

#### 4. Discussion

#### 4.1. Phylogenetic relationships

This study presents the most comprehensive phylogenetic analysis of Menispermoideae to date, based on six DNA regions from the plastid and nuclear genomes (Fig. 2). The inter-tribal relationships within Menispermoideae recovered here are highly congruent with the results of previous studies (Wang et al., 2012; Ortiz et al., 2016). In agreement with other studies (Wang et al., 2007, 2012; Hoot et al., 2009; Jacques et al., 2011; Ortiz et al., 2016), our results also support a non-monophyletic *Cocculus*. For the first time, our phylogeny includes all eight of the currently recognized species of *Cocculus*, which are distributed in four different clades (Fig. 2). Another important finding is that the newly sampled *C. trilobus*  Mayuzumi 3826 and *C. trilobus* Tomoko Fukuda *s.n.* formed a clade nested in *C. orbiculatus*, whereas *C. trilobus* Hong YP H310 is nested in *Pachygone* and sister to *P. sinica*. By carefully checking the morphology of the three accessions, along with examination of the herbarium records, we found that *C. trilobus* Hong YP H310 has characteristics of *P. sinica* and is a misidentified accession. Thus, previous phylogenies where *C. trilobus* was a member of *Pachygone* (Wang et al., 2012; Ortiz et al., 2016) were erroneous due to a misidentified sample of *P. sinica*.

#### 4.2. Biogeography of Pachygoneae

Our two dating analyses generated largely congruent time estimates for Pachygoneae (Figs. 3 and S3). The species-level age estimation suggests a crown group age of 47.65 Ma (95% HPD: 33.33–61.44) for the tribe, which coincides with previous time estimates (Wang et al., 2012; Wefferling and Hoot, 2018). The stem group age of *Hyperbaena* is estimated to be 40.07 Ma (95% HPD: 30.20–52.08), which is highly congruent with the result of Wang et al. (2012; 33.5–54.7 Ma), yet it is much younger than that of Jacques et al. (2011; 64.6 Ma). However, Jacques et al. (2011) only used two plastid loci (*rbcL* and *atp*B) and did not retrieve the monophyly of Pachygoneae. In this study, we included more taxa of Pachygoneae and more characters than in previous studies, which are expected to be able generate more accurate estimates (Yang and Yoder, 2003; Jian et al., 2008). Based on endocarp characters, the extinct species *C. lottii* from the Middle Eocene of the Messel Formation, Germany, is close to *C. carolinus*, *C. orbiculatus*, and *C. hirsutus* (Collinson et al., 2012). This fossil record is in accordance with our time estimate for the most recent common ancestor of these three species (*c.* 44 Ma). These render support to the ages estimated here for Pachygoneae.

Our molecular dating and ancestral area reconstruction analyses indicate that the most recent common ancestor of Pachygoneae was likely present in tropical Asia around the early-middle Eocene boundary (47.65 Ma, 95% HPD: 33.33-61.44; node 3), and subsequently dispersed into the New World by c. 44 Ma (95% HPD: 33.46–56.74; node 4). In the early Cenozoic, the floristic exchange between Eurasia and the New World might have occurred through either the North Atlantic land bridge (NALB) or the Bering Land Bridge (BLB) (Wolfe, 1975; Tiffney, 1985b; Tiffney and Manchester, 2001). The latitude of the NALB was ca. 15° lower than at present and is thought to have maintained sufficient warmth, light, and humidity for thermophilic plants to thrive, whereas the BLB was less likely for thermophilic taxa to disperse across during most of the Cenozoic owing to its high latitude (between 69° to 75° N), limited light availability, and lower temperatures (Tiffney and Manchester, 2001). After the cooling period near the early to middle Eocene boundary, the middle Eocene witnessed a warm interval (~43-46 Ma), but this warm interval was not as warm as in the early Eocene (Fig. 4; Wolfe, 1997; Zachos et al., 2008). The fossil C. lottii, endocarps from Messel in Germany, suggest that Pachygoneae was distributed in Europe in the middle Eocene, although its exact systematic position in side *Cocculus* 

s.l. is unclear (Fig. 1; Collinson et al., 2012). Considering the age estimate and the fossil distribution in Europe, Pachygoneae most likely migrated into the New World via the NALB. Biogeographical analyses indicated that many thermophilic plant lineages, such as *Dendropanax* (Araliaceae; Li and Wen, 2013), *Cinnamomum* (Lauraceae; Huang et al., 2016), and *Meliosma* (Sabiaceae; Yang et al., 2018), might have expanded their ranges during the Eocene via the NALB.

Our divergence time estimates indicate that the clade containing the tropical American Hyperbaena originated at about 40 Ma (node 5 in Fig. 4), which is congruent with a cooling interval in the late Eocene (~38–42 Ma; Wolfe, 1997; Zachos et al., 2008). Li and Wen (2013) suggested that the split of the Neotropical *Dendropanax* and its Asian sister group occurred at the same period (41.83 Ma). The clade now represented by the subtropical North American C. carolinus and C. *diversifolius* diverged from its Asian sister clade later, at about 32 Ma (node 6 in Fig. 4), a period of drastical climatic deterioration (Wolfe, 1997; Zachos et al., 2008). A similar split time was found in Magnoliaceae (30.22 Ma, 95% HPD: 22.20-38.43; Nie et al., 2008) and *Diplazium* (31.2 Ma, 95% HPD: 24.7–38.4; Athyriaceae; Wei et al., 2015). As the climate cooled, thermophilic lineages may have moved to lower latitudes, which could have resulted in the disjunction of the distribution of tropical lineages first, followed by subtropical and temperate lineages, as in Pachygoneae. Thus, our study provides the new evidence for supporting the boreotropical flora hypothesis, in addition to the micro- and macrofossil evidence from the early Eocene

London Clay (Collinson, 1983; Reid and Chandler, 1933) and the middle Eocene Clarno Formation in northcentral Oregon (Manchester, 1994).

Our results suggest that *Pachygone* arrived in Australasia from Asia in the early to late Miocene (c. 20-8 Ma, between nodes 7 and 8 in Fig. 4). The collision between Asia and New Guinea/Australian plates occurred about 25 Ma (Morley, 1998; Hall, 2002, 2011), and land connection between these two regions became continuous gradually as the convergence of the Sahul and Sunda shelves, c. 12 Ma (Baldwin et al., 2012; Crayn et al., 2014). The Asia-Australasia floristic exchange, especially of rain forest lineages, became increasingly likely since the middle Miocene (Crayn et al., 2014; Wu et al., 2019), as a result of a climate amenable to megathermal forest expansion, the close proximity between Sahul and Sunda, and the emergence of land to support stepping-stone migration. Our analysis on Tinospora, another genus of Menispermaceae, indicates a similar dispersal timing (Lian et al., 2019). In addition to Pachygone and Tinospora, several other megathermal plant taxa, such as Livistona (Arecaceae; Bacon et al., 2012), Cinnamomum (Lauraceae; Huang et al., 2016), and Dendrocnide (Urticaceae; Huang et al., 2019), represent similar eastward migration pattern. These studies support the hypothesis that the exchange between Asia and Australasia was strongly asymmetrical, i.e. "west-to-east" migration predominated over the reverse (Crayn et al., 2014; Wu et al., 2019).

#### 4.3. Taxonomic implications

Molecular and morphological evidence indicates the need to reinstate *Cebatha* and *Nephroia*, establish a new tribe for *Cebatha*, and transfer *C. laurifolius* to *Pachygone*. Summary of the key taxonomic revisions in this study is shown in Fig. 5. The geographic distribution of the new delimited Pachygoneae is shown in Fig. S5. Further details about the required taxonomic changes are presented below.

The reinstatement of Cebatha and proposal for a new tribe Cebatheae. In 1775, Forsskål (1775) established two genera Cebatha and Leaeba, but did not place any species in them. One year later, Forster and Forster (1776) established the genus Epibaterium to include the sole species E. pendulum, which was reduced to Cocculus by de Candolle (1818), i.e. C. pendulus. Kuntze (1891) reduced Leaeba, Epibaterium, and Cocculus to Cebatha. However, Diels (1910) used Cocculus as the generic name, which was accepted by subsequent authors (e.g., Troupin, 1962; Forman, 1974b; Kessler, 1993; Luo et al., 2008). Our phylogenetic analyses indicate that C. pendulus and C. balfourii (Cocculus 1) do not cluster with other Cocculus species (Fig. 2). These two species are markedly different from Cocculus 2 (C. hirsutus, the type of *Cocculus*) in having the pericarp of dried fruit being easily removed (vs. hard to remove), glabrous and entire petals (vs. pubescent and bifid or deeply emarginate), and imperforate condyle (vs. perforate) (Diels, 1910; Troupin, 1962; Forman, 1974b; Jacques, 2009). In addition, endocarps of C. pendulus lack a sharp dorsal ridge, whereas endocarps of C. balfourii and C. hirsutus have a wing-like dorsal ridge, but endocarps of C. balfourii and C. hirsutus are different in lateral ridges (unbranched vs. branched or inconspicuous) (Forman, 1974b). *Cocculus pendulus* and *C. balfourii* also differ from remaining Asian/American *Cocculus* (*Cocculus* 3 and 4; Fig. 2) in many morphological characters, such as entire petals (*vs.* bifid or deeply emarginate), a gynoecium consisting of three carpels (*vs.* six), and endocarps lacking or with a few transverse ridges (*vs.* numerous) (Diels, 1910; Forman, 1974b). Therefore, our data support the resurrection of *Cebatha* to embrace two species, *C. pendula* and *C. balfouriana*.

Our phylogenetic analyses indicate that *Cocculus* 1 is in an unresolved position in relation to Pachygoneae, Tiliacoreae, Spirospermeae, and Cissampelideae (Fig. 2). These five lineages diverged rapidly over a period shorter than 6 million years (Myr), and perhaps in as short as 1 Myr (Fig. 3). It is not reasonable to include *Cebatha* in any of the already recognized tribe, thus we propose a new tribal name for the genus:

Cebatheae Lian Lian & Wei Wang, trib. nov. TYPE: *Cebatha* Forsk. Fl. Aegypt. -Arab.: 171. 1775.

Lianas, dioecious. Leaves ovate, trapezoid or spine-like. Flowers minute. Male flowers: sepals 6, ovate-elliptic, fleshy or membranous; petals 6, ovate-obovate, glabrous; stamens 6. Sepals and petals are the same as male flowers. Drupe reddish when fresh but turning black when dried, pericarp of dried fruit easily rubbed off. Endocarp with no lateral ridges or no more than 15, not perforated in the center.

*The reinstatement of Nephroia.* In 1790, Loureiro established the genus *Nephroia* to include the one species *Nephroia sarmentosa*. Diels (1910) reduced *Nephroia* to

Cocculus, i.e. Cocculus sarmentosus, and he also thought that C. orbiculatus was a synonym of C. trilobus. Forman (1962) accepted C. trilobus as including C. sarmentosus and C. orbiculatus. However, Forman (1968) suggest that C. orbiculatus is the valid name, instead of C. trilobus. Our phylogenetic analyses indicate that C. orbiculatus, C. trilobus, C. carolinus and C. diversifolius form a clade (Cocculus 3), which has a distant relationship with C. hirsutus (Fig. 2). Cocculus 3 markedly differ from C. hirsutus in having glabrous sepals (vs. pubescent), six carpels (vs. three), and endocarps without a dorsal ridge (vs. a sharp dorsal ridge) (Diels, 1910; Forman, 1974b). In addition, the species in *Cocculus* 3 are restricted to forests, whereas Cocculus 1 and 2 clades inhabit dry and open areas. Thus, we resurrected Nephroia as a distinct genus and assigned N. orbiculata as its type. Our analyses further show that C. trilobus is embedded within C. orbiculatus, which supports the synonymy of C. trilobus under C. orbiculatus (Forman, 1968; Luo et al., 2008). Thus, the resurrected Nephroia contains three species, N. orbiculata, N. carolina and N. diversifolia. The necessary taxonomic changes are proposed here.

*Nephroia orbiculata* (L.) Lian Lian & Wei Wang, **comb. nov.**  $\equiv$  *Menispermum orbiculatum* L. in Sp. Pl. 1: 341. 1753.  $\equiv$  *Menispermum trilobum* Thunb. in Fl. Jap. 194. 1784.  $\equiv$  *Cocculus orbiculatus* (L.) DC. in Syst. I: 523. 1817. TYPE: "Cocculi Orientalis Frutex, convolvulaceus, orbiculatis foliis, prona parte villosis, ex una de Insulis Crocodilorum" in Plukenet, Amalth. Bot., 61, t. 384, f. 6, 1705 (lectotype designated by Troupin in Bull. Jard. Bot. État Bruxelles 25: 141. 1955). = Nephroia

sarmentosa Lour. in Fl. Cochinch. 2: 692. 1790. TYPE: Cochinchina, Loureiro 565 (holotype, BM-000554339, [image!])

*Nephroia carolina* (L.) Lian Lian & Wei Wang, **comb. nov.**  $\equiv$  *Menispermum carolinum* L. in Sp. Pl. 1: 340. 1753.  $\equiv$  *Cocculus carolinus* (L.) DC. in Syst. I: 524. 1818.  $\equiv$  *Wendlandia caroliniana* (L.) Nutt., Gen. N. Amer. Pl. 1: 241. 1818.  $\equiv$ *Cebatha carolina* (L.) Britton, Mem. Torrey Bot. Club 5: 162. 1894.  $\equiv$  *Epibaterium carolinum* (L.) Britton, Ill. Fl. N. U.S. (Britton & Brown), ed. 2, 2: 131. 1913. TYPE: U.S.A. South Carolina, Williamsburg Co., 5 miles southeast of Gourdin, 10 Jul 1939, *R.K. Godfrey & R.M. Tryon 373* (BH, neotype designated by Reveal & Jarvis 2009: 980, not seen; isoneotype, GH-00257737, not seen).

*Nephroia diversifolia* (DC.) Lian Lian & Wei Wang, **comb. nov.**  $\equiv$  *Cocculus diversifolius* DC. in Syst. I: 523. 1818.  $\equiv$  *Cebatha diversifolia* (DC.) Kuntze, Revis. Gen. Pl. 1: 9. 1891.  $\equiv$  *Epibaterium diversifolium* (DC.) Tidestr., Proc. Biol. Soc. Washington 48: 39. 1935. TYPE: DC. plate 10 deposited in G, including two drawings copied from Plates 1490 and 1519, Torner Collection of Sessé and Mociño Biological Illustrations (lectotype designated by McVaugh 2000: 391).

*The inclusion of C. laurifolius* in *Pachygone. Cocculus laurifolius* was first described by de Candolle (1818). However, de Candolle (1818) also noted that this species was unique in that the base of the petal does not embrace the filament. In agreement with the results of previous studies (Wang et al., 2007, 2012; Ortiz et al., 2016), our analyses also indicate that *C. laurifolius* is nested in *Pachygone* and is

sister to *P. valida. Cocculus laurifolius* shares a few morphological similarities with *Pachygone*, including a gyneocium with three carpels and an endocarp lacking transverse ridges (Diels, 1910; Jacques, 2009). Thus, our results suggest that *C. laurifolius* is a member of *Pachygone*. A new combination is made here.

Pachygone laurifolia (DC.) Lian Lian & Wei Wang, comb. nov.  $\equiv$  Cocculus laurifolius DC. in Syst. I: 530. 1818.  $\equiv$  Menispermum laurifolium (DC.) Roxb., Fl. Ind. 3: 815. 1832.  $\equiv$  Nephroica laurifolia Miers, Ann. Mag. Nat. Hist. ser. 2, 7(37): 42. 1851.  $\equiv$  Holopeira laurifolia (DC.) Miers, Ann. Mag. Nat. Hist. ser. 3, 19(109): 29. 1867.  $\equiv$  Cebatha laurifolia (DC.) Kuntze, Revis. Gen. Pl. 1: 9. 1891. TYPE: India (holotype, G-00201635 [image!]).

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**Fig. 1.** Geographic distribution of Pachygoneae. The number of species sampled in this study/total numbers of species are given in parentheses. Red star denotes the location of the fossil *Cocculus lottii*, endocarps from the early Middle Eocene of Germany (Collinson et al., 2012).



Fig. 2. ML tree inferred from the combined six plastid and nuclear dataset. Numbers above branches are bootstrap values (BS  $\geq$  60%) and posterior probabilities (PP  $\geq$  0.80). An asterisk (\*) indicates BS = 100% or PP = 1.0. Tribes of Ortiz et al. (2016) and the new proposed tribe Cebatheae are indicated on the right.



**Fig. 3.** Chronogram of Menispermoideae with 50 taxa based on the six-locus dataset using BEAST. Gray bars represent 95% highest posterior density intervals. The  $C_1$ - $C_6$ indicate the placements of fossil calibration points (see Table S2 for details). Nodes of interests were marked as 1–8. The grey area shows that the five tribes have diversified rapidly over a period of < 6 million years. Pal = Paleocene; Oli = Oligocene; Pl = Pliocene.



**Fig. 4.** Ancestral range reconstruction (ARR) using Lagrange, modified from Fig. S4. Global temperature curve is modified from Zachos et al. (2008). Labeled nodes (3 to 8, as referred to Fig. 3) are discussed in the text. The AARs with the highest likelihood are shown as colored boxes at each node. Single area boxes indicate an ancestor confined to a single geographic area; combined boxes indicate an ancestor with a distribution encompassing two areas; two boxes separated by a space indicate the ancestral ranges inherited by each of the daughter lineages arising from the node.

Numbers near the nodes indicate the highest ML probabilities. Pal = Paleocene; Pl = Pliocene.



Fig. 5. Summary of the phylogenetic relationships in Pachygoneae as revealed by our molecular and as proposed to achieve mutually monophyletic genera. Dashed line indicates the node with BS < 60% and PP < 0.80. The number of species are given in parentheses.