Molecular Phylogenetics and Evolution 152 (2020) 106918

Contents lists available at ScienceDirect

Molecular Phylogenetics and Evolution

journal homepage: www.elsevier.com/locate/ympev

Shining a light on species delimitation in the tree genus *Engelhardia* Leschenault ex Blume (Juglandaceae)

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ARTICLE INFO

Keywords:
Species delimitation
Integrative taxonomy
Engelhardia
Morphology
Tropical and subtropical Asia

ABSTRACT

Enhanced efficacy in species delimitation is critically important in biology given the pending biodiversity crisis under global warming and anthropogenic activity. In particular, delineation of traditional classifications in view of the complexity of species requires an integrative approach to effectively define species boundaries, and this is a major focus of systematic biology. Here, we explored species delimitation of *Engelhardia* in tropical and subtropical Asia. In total, 716 individuals in 71 populations were genotyped using five chloroplast regions, one nuclear DNA region (nrITS), and 11 nuclear simple sequence repeats (nSSR). Phylogenetic trees were constructed and relationships among species were assessed. Molecular analyses were then combined with 14 morphological characteristics of 720 specimens to further explore the species boundaries of *Engelhardia*. Integrating phylogenetic and morphological clusters provided well-resolved relationships to delineate seven species. The results suggested that: first, that *E. fenzelii*, *E. roxburghiana*, *E. hainanensis*, *E. apoensis*, and *E. serrata* are distinct species; second, *E. spicata* var. *spicata*, *E. spicata* var. *aceriflora*, *E. spicata* var. *colebrookeana*, and *E. rigida* should be combined under *E. spicata* and treated as a species complex; third, *E. serrata* var. *cambodica* should be raised to species level and named *E. villosa*. We illuminated that bias thresholds determining the cluster number for delimiting species boundaries were substantially reduced when morphological data were incorporated. Our results urge caution when using the concepts of subspecies and varieties in order to prevent confusion, particularly with respect to species delimitation for tropical and subtropical species. In some cases, re-ranking or combining subspecies and/or varieties may enable more accurate species delimitation.

1. Introduction

Species are the fundamental units of biology, providing the most practical metric for distinguishing habitats and tracking the progress of Earth's biodiversity (Costello et al., 2013). Therefore, effective recognition of species is the first step in the fields of phylogeny, evolution, biogeography, and biodiversity conservation (de Queiroz, 2005; Mayr, 1982). However, mistakes are inevitable when determining species, and can result in erroneous interpretations in research that uses species-based information. In particular, mistakes of plant identification in tropical regions are very common (Goodwin et al., 2015) and can adversely affect recognition and understanding of species diversity in global biodiversity hotspots. Moreover, associated biased factors may result in higher costs or unpredictable waste of effort in species and/or biodiversity conservation (Su et al., 2015).

Indeed, previous research has emphasised that use of only a single line of evidence to delimit species may result in the detection of more or fewer species than are actually present (Edwards and Knowles, 2014).

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https://doi.org/10.1016/j.ympev.2020.106918
Received 12 November 2019; Received in revised form 16 July 2020; Accepted 21 July 2020
Available online 30 July 2020

Perspectives on morphological classification are often biased by researcher preferences or weighting characteristics. Widespread species tend to be more morphologically diverse compared with narrowly endemic species, which can easily lead to more varieties (Darwin, 1859). Moreover, similar traits can appear in lineages that are not closely related owing to parallel evolution (Schluter et al., 2004), which leads to distinct lineages clustering together. Although phylogenetic analyses can substantially enhance our understanding of the relationships among species, they do not provide a complete solution to species delimitation, as the number and nature of clusters often depend on arbitrary thresholds or parameters (Posso-Terranova and Andrés, 2018). Owing to the shortcomings of using a single parameter (e.g., morphological or molecular data) to delimit species boundaries, an integrative approach should be developed as a major tool in modern systematics (Wiens, 2007). The rationale for this is that a separate and evolving metapopulation lineage is the primary property defining species, and integration of multiple operational approaches (morphology, genetics, etc.) to define and validate this property can increase the efficiency and accuracy of species delimitation greatly (de Queiroz, 2007). Recently, integrative methods, such as the multivariate clustering of morphological, genetic data, have helped define species boundaries in animals and plants (Carstens and Satler, 2013; Damasco et al., 2019; Misiewicz and Fine, 2014; Posso-Terranova and Andrés, 2018; Prata et al., 2018).

However, species delimitation within the family Juglandaceae remains a challenge, as hybridization and frequent gene flow have commonly occurred in its complex evolutionary history (Bai et al., 2014; Dong et al., 2017; Zhao et al., 2018). In particular, apparently continuous species intergradations are problematic among the well-studied temperate groups in the family (Kozlowski et al., 2018; Stone et al., 2009). Moreover, delimitation of tropical species remains daunting because of the extent of plant diversity in the tropics and the paucity of comprehensive floristic accounts (Ulloa, 2017), with on average more than 50% of tropical specimens likely to be identified incorrectly (Goodwin et al. 2015). Thus, integrative methods and comprehensive sampling are needed to relieve the problems with the delimitation of tropical and subtropical Juglandaceae species.

Engelhardia is a genus of deciduous or evergreen trees in the walnut family (Juglandaceae) and is considered to be one of the primitive genera of the tribe Engelhardieae (Song et al., 2020). The genus occurs in tropical and subtropical East Asia, the Indo-China Peninsula, and the Malay Archipelago; while the tribe also contains *Oreomunnea* and *Alfaroa*, both of which are distributed in Central America. The taxonomy and phylogeny of *Engelhardia* have been explored for decades, but are still subjects of disagreement (Manos and Stone, 2001; Manos et al., 2007; Stone, 2010). In particular, *Engelhardia* remains poorly understood due to inaccessibility of study material, large ocean separation, and vast latitudinal distribution in tropical and subtropical Asia.

A relatively comprehensive classification of the genus *Engelhardia* was conducted by Manning (1966) based on herbarium materials, but there was a lack of comprehensive field investigations and molecular analyses. Most of the studies related to *Engelhardia* have focused mainly on fossils (Hermsen and Gandolfo, 2016; Manchester, 1987; Manchester et al., 1994; Meng et al., 2015), or taxonomic affinities at higher levels such as the tribe or family (Manos and Stone, 2001; Manos et al., 2007). Consequently, *Flora of China* (FOC) indicated that the number of species of *Engelhardia* is an open question and the taxonomy of the genus suffers from a lack of good specimens across its vast geographic range (Lu et al., 1999). Moreover, the taxonomy of *Engelhardia* is complicated further by the use of multiple synonymous names in different areas (Manning, 1966; Sidiyasa, 2015).

Additionally, previous taxonomy has been mainly focused on morphological traits such as inflorescences and leaflets, which has led to the proposal of subdivisions in *Engelhardia* (e.g., Manning, 1966). Five species of *Engelhardia* collected across the entire distribution area were identified with this focus: the widely distributed *E. roxburghiana*, *E. spicata*, and *E. serrata*, and the more narrowly endemic *E. rigida* and *E. apoensis*. In addition, some varieties have been recognised by Jacobs (1960) and Manning (1966). The species listed in FOC are somewhat different: *E. roxburghiana* (including *E. fenzelii*), *E. hainanensis*, *E. spicata* (including *E. spicata* var. *aceriflora*, *E. spicata* var. *colebrookeana*), and *E. serrata* var. *cambodica* (Lu et al., 1999). Moreover, the taxonomic placement of *E. roxburghiana* is controversial, with Iljinskaya (1993) proposing that it should be considered as a new monotypic genus, *Alfaropsis*. However, *Alfaropsis* was considered to be synonymous with *Engelhardia* (Lu et al., 1999), and its position remains unresolved (Manos and Stone, 2001; Manos et al., 2007; Stone, 2010). It is also notable that *E. roxburghiana*, *E. spicata*, and the varieties occur in mixed communities, and there may be hybridization among them. *E. apoensis* is probably the rarest species of *Engelhardia*, having been collected only 12 times, and *E. serrata* has included apprently excessive numbers of varieties in previous research (Manning, 1966). These aspects of *Engelhardia* taxonomy also require further investigation. The characteristics and distributions of previously recognised *Engelhardia* taxa are provided in Table S1 and Fig S1.

Accordingly, this study aims to explore species delimitation within *Engelhardia* using evidence from integrative chloroplast DNA (cpDNA), nuclear ribosomal DNA (nrDNA), nuclear simple sequence repeats (nSSR) analyses, and morphology across its entire geographic distribution in tropical and subtropical Asia. Our results enabled us to, (1) provide insight into species concepts and delimitation within *Engelhardia*; (2) explore integrative approaches, particularly methods involving integrating molecular and morphological data to define species boundaries; and (3) reveal how many species within *Engelhardia*.

2. Materials and methods

2.1. Sample collection

Sampling was undertaken for *Engelhardia* taxa recognised by Manning (1966), the FOC (Keren and Lu, 1979; Lu et al., 1999), *Flora Malesiana* (Jacobs, 1960), and websites such as the CVH (Chinese Virtual Herbarium: http://www.cvh.ac.cn/class), POWO (Plants of the World Online: http://powo.science.kew.org), Tropicos (http://www.tropicos.org), and the GBIF (Global Biodiversity Information Facility: https://www.gbif.org). A total of 716 individuals of *Engelhardia* were collected from 71 populations, representing ten taxa from across tropical and subtropical Asia (Fig. 1, see Table S2 for the geographic coordinates). Our sampling scale covered almost the entire distribution of *Engelhardia* from south of the Yangtze River to Indonesia. However, we did not sample from Nepal, the Philippines, the Malay Peninsula, and New Guinea. The nrDNA and cpDNA sequence variation and nSSR analyses were performed using genetic materials from each sample collected.

2.2. DNA fragments and nSSR sequenced

Total genomic DNA was extracted using a Plant Genomic DNA Kit (Tiangen Biotech, China). Five cpDNA regions (*psb*A-*trn*H, *trn*L-*trn*F, *rps*16, *trn*S-*trn*G, and *rpl*32-*trn*L), and the nrITS region, were sequenced (Table S3). In addition, 11 selected nSSR loci were amplified: HQ23, HQ49, HQ54, HQ89, JC4833, JC6576, WGA27, WGA79, WGA089, WGA202, and WGA321. Detailed information on lab protocols is provided in Table S4. All targeted chloroplast sequences were concatenated and edited manually using Geneious v6.1.2 (https://www.geneious.com/).

2.3. Network and phylogenetic analyses of DNA sequences

The combined cpDNA haplotypes (*H*) and nrDNA ribotypes (*R*) were analysed using DNASP v6 (Rozas et al., 2017), with the lineage relationships between the haplotypes and ribotypes inferred by median-joining network as implemented in NETWORK v2.0 (Bandelt et al., 1999) and Splits Tree v4.14.8 (Huson and Bryant, 2006). Both plastid DNA and ITS data sets were subjected to Bayesian analyses using

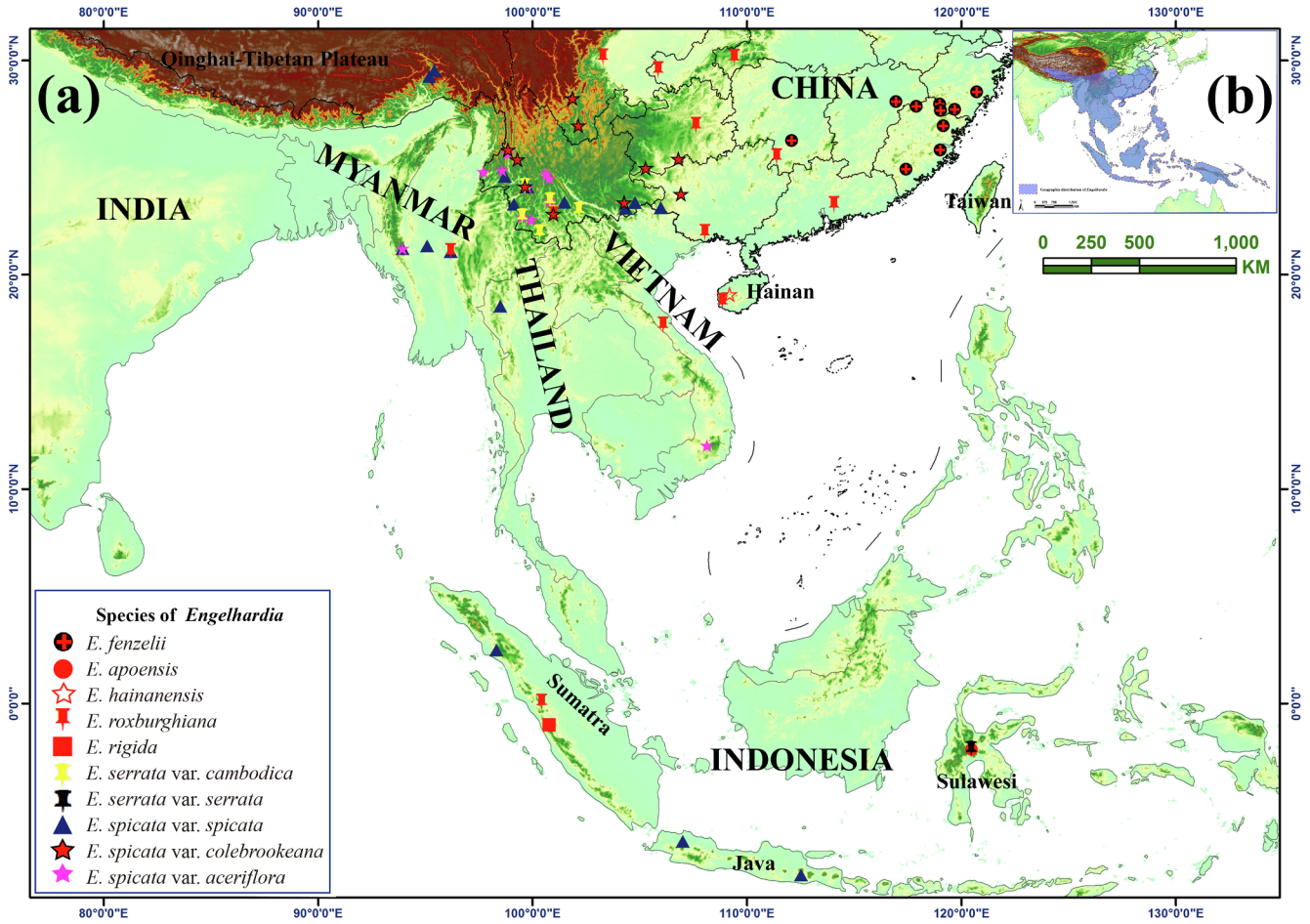

Fig. 1. (a) The geographic distribution of *Engelhardia*. A total of 71 locations were collected across tropical and subtropical Asia. (b) The blue shadows denote the entire geographic distribution areas of *Engelhardia* (adopted from Meng et al., 2015; Manchester, 1987). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

MrBayes v3.1.2 (Huelsenbeck and Ronquist, 2001), and maximum-likelihood (ML) analyses were performed in RAxML-HPC BlackBox via the CIPRES portal (Miller et al., 2010). Following the phylogeny for Juglandaceae, *Rhoiptelea chiliantha* was selected as outgroup (Manos and Stone, 2001; Manos et al., 2007). The best-fit evolutionary model and gamma rate heterogeneity were chosen by running the datasets using the Akaike information criterion (AIC) with PAUP* v4.0b10 (Swofford, 2002) and Modeltest v3.7 (Posada and Crandall, 1998). The best-fit substitution models suggested that the combined cpDNA and nrDNA data were TIM + I + G and GTR + I + G, respectively. For Bayesian analyses, the Markov chain Monte Carlo (MCMC) algorithm was run for 5×10^6 generations with one cold and three heated chains, starting from random trees and sampling one out of every 500 generations. Examination of the log-likelihood values suggested that stationarity was reached in about 5×10^5 generations. For ML analyses, the confidence levels of the nodes supporting the trees were determined using the fast bootstrapping option with 1000 bootstrap replicates.

2.4. Neighbour-joining (NJ) phylogenetic analyses and population genetic structure based on nSSR

Microsatellite data (nSSR) were edited and formatted in GenAlEx v6.3 (Peakall and Smouse, 2012) (see Table S5 for the data). The phylogenetic relationships of the sampled populations were determined using the NJ method with Powermarker v3.25 (Liu and Muse, 2005). Inference of genetic structure from the microsatellite data was conducted with Structure v2.3 (Pritchard et al., 2003). The simulation was run with a cluster number (K) ranging from 1 to 20 for each set. Each run consisted of a burn-in of 2×10^4 iterations, followed by 10^5 iterations. Results and convergence of the MCMC procedure were subject to repeated testing by carrying out a series of 10 replicate runs for each K-prior value. The K-prior value was evaluated in log-likelihood form using Structure Harvester v0.6.8 (Earl and von Holdt, 2012).

2.5. Statistical analyses of morphology

Basic morphological information was also obtained by measuring the specimens with flowers and seeds during the field survey, as well as herbarium specimens from the herbaria (Tables S6 and S7). We first observed and measured 25 characteristics from 720 individuals, including 13 quantitative traits and 12 qualitative traits (Table S8), based on important morphological features from the literature (Jacobs, 1960; Keren and Lu, 1979; Lu et al., 1999; Manning, 1966; Manos and Stone, 2001; Stone, 2010). To determine which traits provided useful information, we examined statistically significant morphological differences using AMOVA (Table S6). A total of 25 morphological characteristics were used to test placement of the specimens in the multivariate space based on Discriminant Analysis. Data collected from the field were selected to estimate the morphological differences among populations and compared against the descriptions available for each taxon (Li et al., 2019), with highly labile population traits removed from the analyses. Finally, a total of 14 characters were used for principal components analysis (PCA, Wold et al., 1987). All calculations

were performed in the IBM SPSS Statistics v22.0 statistical software package (SPSS, Armonk, NY: IBM Corp).

2.6. BPP and iBPP analyses

Molecular fragments were also used to test species delimitation hypotheses with the model-based species delimitation program BPPX v1.2.2 (Rannala and Yang, 2003; Yang and Rannala, 2010). We used uniform rooted trees that were used as the species model prior, and the parameter set as ancestral population size θ and tree age τ: θ = G(1, 10) and τ = G(1, 10); θ = G(1, 10) and τ = G(2, 2000); θ = G(2, 2000) and τ = G(1, 10); θ = G(2, 2000) and τ = G(2, 2000). The MCMC chains for 5×10^5 generations with parameter samples were taken every five generations, with a burn-in period of 2×10^5 generations. (Sukumaran and Knowles, 2017; Yang et al., 2019). A joint Bayesian inference based on molecular and morphological data was used to analyse species delimitation hypotheses by integrative Bayesian Phylogenetics and Phylogeography (iBPP). The parameters θ and τ were also applied in four different prior combinations (as for BPP). A non-informative conjugate prior distribution with parameters $\nu_0 = 0$ and $\kappa_0 = 0$ was used for the trait variances and ancestral means. The MCMC chains for 5×10^5 generations with parameter samples were taken every five generations with a burn-in period of 10^5 generations (Solis-Lemus et al., 2015).

3. Results

3.1. The network and phylogeny of cpDNA haplotypes and nrDNA ribotypes

A total of 716 samples were sequenced, and we obtained 687 combined cpDNA sequences and 659 ITS sequences. The five aligned cpDNA spacers consisted of 4647 base pairs and a total of 39 haplotypes were determined from all individuals sampled (Fig. S2). The nrITS consisted of 755 base pairs and 32 ribotypes were determined (Fig. S3). All sequences are deposited in Genbank, and the accession numbers are MN307497—MN307736. The basic haplotype and ribotype information of the 71 populations is summarised in Tables S2 and S9. The relationships of *E. rigida* and *E. spicata* varieties were more complicated than those of the other taxa (Fig. 2). The geographic distribution of cpDNA haplotypes and nrDNA ribotypes showed *E. hainanensis* is endemic to Hainan Island. *E. fenzelii* is only distributed in the southeast of China. Indonesian endemic species included *E. rigida* and *E. apoensis*, whereas the rest of the sampled species were widespread (Figs. S2 and S3).

In general, the multi-locus DNA analyses provided a well-resolved phylogenetic backbone for the major clades of *Engelhardia*. The topologies of the phylogenetic trees based on Bayesian and ML methods were nearly identical (Figs. 3 and S4), with no major topology conflicts between the cpDNA tree and the nrDNA tree, and the results revealed seven clades (Fig. 3). A deep split in the sample identified branches leading to subclades of *E. roxburghiana* and *E. fenzelii* as sister lineages, which was distinctive to a larger clade containing the remaining species of *Engelhardia*. This was well supported in the ITS and cpDNA trees, supporting the subclades of *E. roxburghiana* and *E. fenzelii* as two species. The clades recovered in the ITS and cpDNA analyses indicated that *E. apoensis* + *E. serrata* var. *serrata* was sister taxon to *E. serrata* var. *cambodica*. In contrast, *E. spicata* var. *spicata*, *E. spicata* var. *aceriflora*, *E. spicata* var. *colebrookeana*, and *E. rigida* formed a complex, intermixed clade in both analyses. In addition, *E. hainanensis* was a well-supported sister taxon to the *E. spicata* complex (Fig. 3).

3.2. Genetic clustering and structure of nSSR

The NJ tree based on 11 microsatellite loci produced similar results to the phylogenetic trees based on cpDNA and nrDNA, with seven clearly defined clades: (1) *E. roxburghiana*; (2) *E. fenzelii*; (3) *E. hainanensis*; (4) *E. apoensis*; (5) *E. serrata* var. *serrata*; (6) *E. serrata* var. *cambodica*; and (7) the *E. spicata* var. *spicata*, *E. spicata* var. *aceriflora*, *E. spicata* var. *colebrookeana*, and *E. rigida* complex (Fig. 4). Uneven sampling can often lead to erroneous inferences with respect to hierarchical structure and downward-biased estimates of the true numbers of subpopulations. In particular, distinct subpopulations that have been under-sampled tend to merge together, while individuals from more extensively sampled subpopulations are generally subdivided into more clusters (Puechmaille, 2016). To avoid such errors in sampling bias and clearly identify clades for controversial species, we divided all of the populations into three groups to estimate their genetic structure. The first group included *E. roxburghiana* and *E. fenzelii* (Fig. S5 and Table S10). The second group included *E. spicata* var. *spicata*, *E. spicata* var. *aceriflora*, *E. spicata* var. *colebrookeana*, and *E. rigida* (Fig. S6 and Table S10). The third group included *E. hainanensis*, *E. apoensis*, *E. serrata* var. *serrata*, and *E. serrata* var. *cambodica* (Fig. S7 and Table S10). With this division, the structure showed that *E. roxburghiana* and *E. fenzelii* were separate species with few gene mixtures between them (Fig. S5). The genetic structure of *E. rigida* was identical to that of *E. spicata* var. *spicata*, *E. spicata* var. *aceriflora*, and *E. spicata* var. *colebrookeana* (Fig. S6). *E. apoensis* and *E. serrata* var. *serrata* showed a similar genetic structure. In contrast, *E. hainanensis and E. serrata* var. *cambodica* displayed distinctly different genetic plots (Fig. S7).

3.3. Morphological clustering

Morphological traits from 720 specimens were explored using PCA (Fig. 5a) and Discriminant Analysis (Fig. 5b). The first three principal components identified by PCA accounted for 70.55% of the variation across all characters, with the first principal components explaining 43.49% of the variation (Fig. 5a). The morphological traits aligned to the first PCA axis (with an absolute value score > 0.5) were fruit hairs, inflorescence of old/new branches, terminal/lateral inflorescences, terminal bud hairs, number of leaflets, branchlet hairs, petiole length, leaflet hairs, and leaflet arrangement. The second principal component explained 15.58% of the total morphological information and included the traits were leaflet hairs, leaflet apex, leaflet thickness, and leaflet length/width ratio. The third principal component explained 11.48% of the information and was associated with twigs colour, leaflet arrangement, and leaflet margins. The PCA identified seven distinct groups from all species of *Engelhardia*, in which all species were well-identified except for *E. rigida* and three varieties of *E. spicata*. *E. roxburghiana* was most similar to, but still distinct from *E. fenzelii*. *E. apoensis*, *E. serrata* var. *serrata*, *E. hainanensis*, and *E. serrata* var. *cambodica* all exhibited clear species boundaries based on these morphological traits (Fig. 5a).

In Discriminant Analysis, the Group Centroid showed that the groups were separated from each other except the species of *E. rigida* and three varieties of *E. spicata* (Fig. 5b). The analysis of these characters produced a good discriminant function, with a total of 88.0% of original grouped cases and 84.8% of cross-validated grouped cases correctly classified (Table S6).

3.4. Species tree inference

The Bayesian species delimitation analyses of the molecular data by BPP. And combined molecular and morphological data were explored by iBPP. These two methods independently gave posterior probabilities of 1.0, indicating strong support for seven species-level clades within *Engelhardia*. Regardless of genetic data alone or combined data, the results supported (1) the separation of *E. fenzelii* from *E. roxburghiana*; (2) the status as independent species of *E. hainanensis*, *E. apoensis*, *E. serrata* var. *serrata*, and *E. serrata* var. *cambodica*; (3) The combination into a single species, without clear infrataxa, of the others (*E. rigida* and the three *E. spicata* varieties) (Fig. 6).

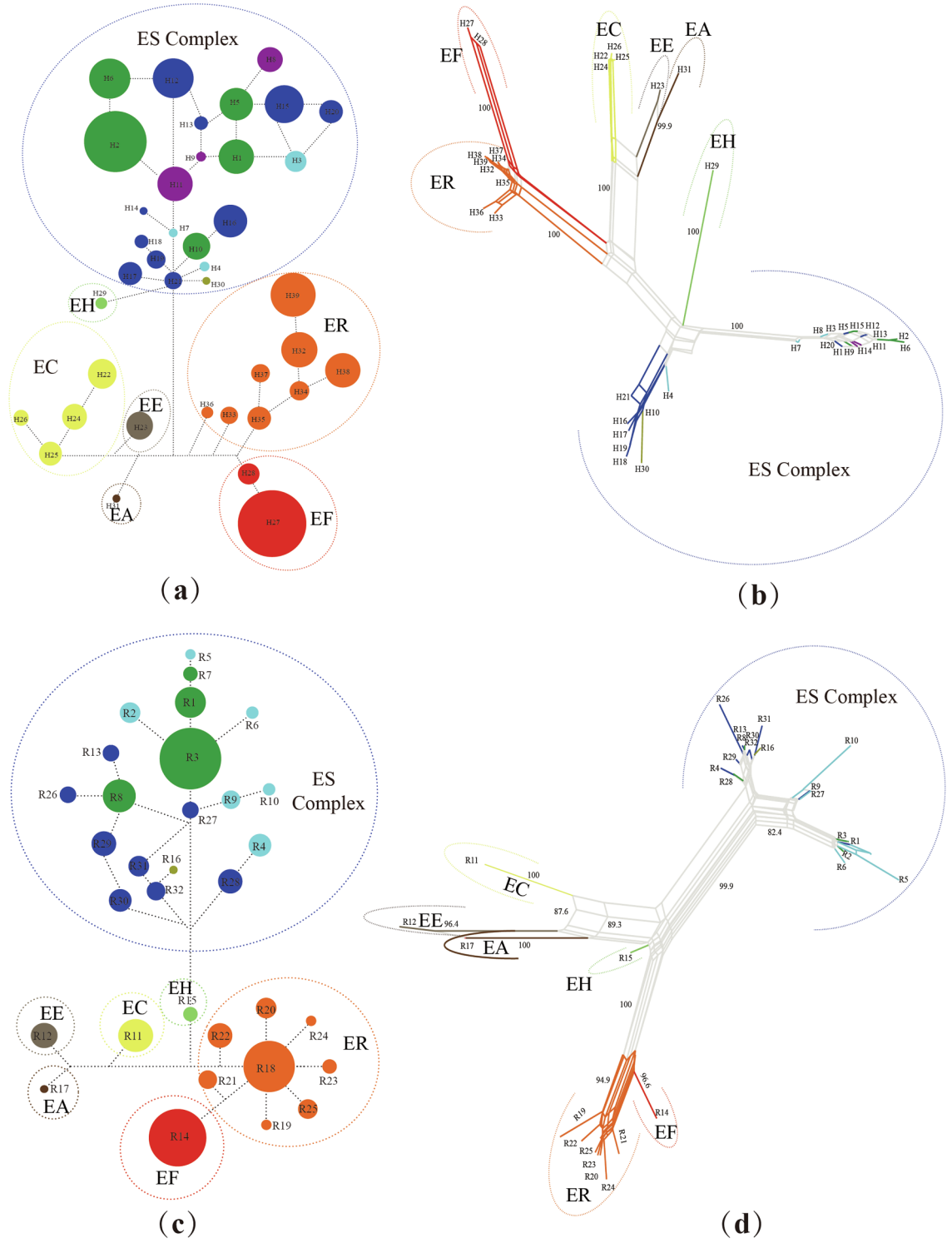

Fig. 2. Networks of *Engelhardia*. (a and c) Estimates from NETWORK. Each circle indicates a single haplotype and ribotype (a and b from cpDNA; c and d from nrITS) sized in proportion to its frequency. (b and d) Estimates from Splits Tree. The number of major branches showing bootstrap support values (> 90% values).

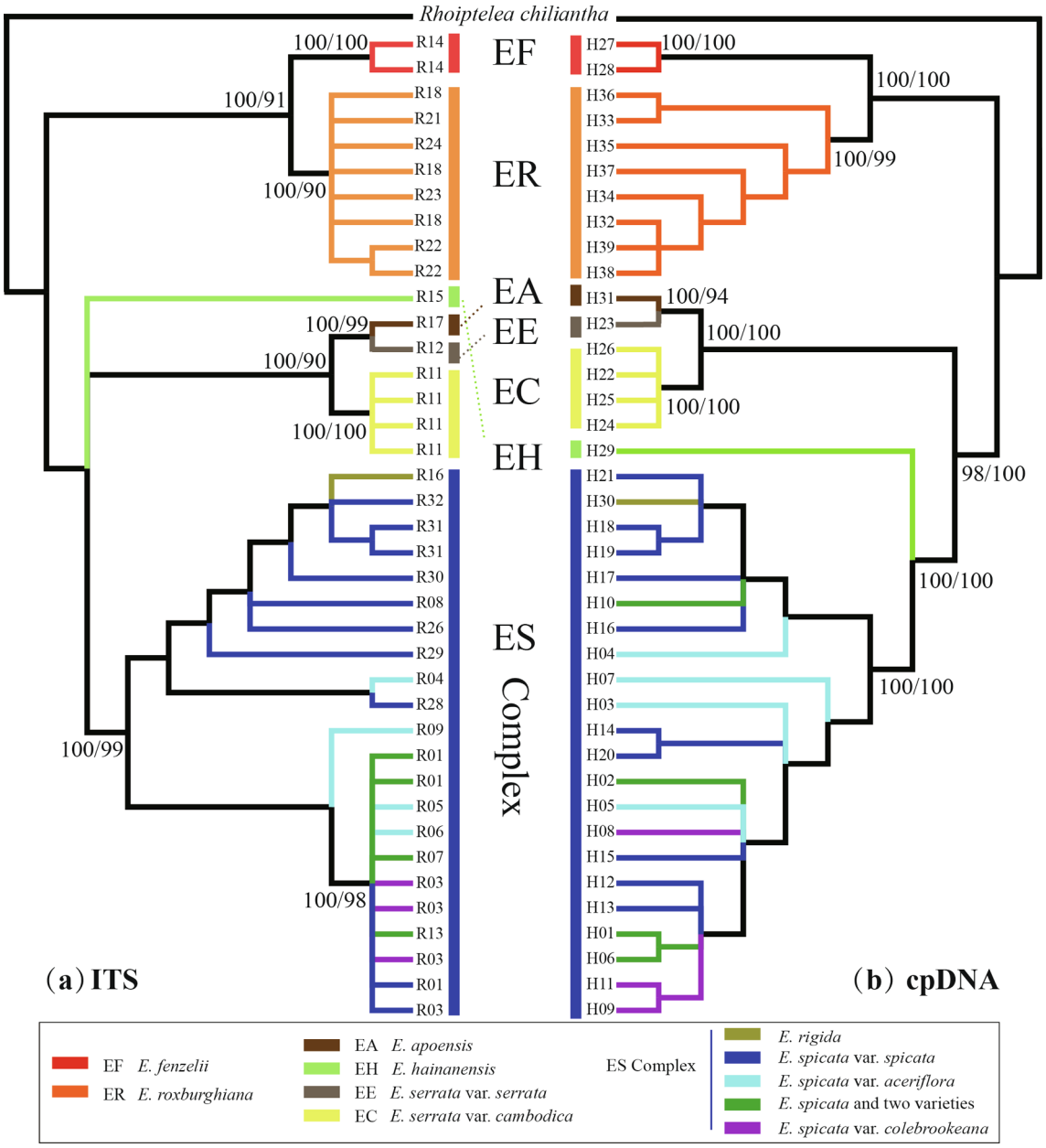

Fig. 3. Bayesian consensus tree based on nrDNA ITS (a; left) and the combined cpDNA haplotypes (b; right). Posterior probability support of Bayesian (before) and bootstrap support values from ML analyses (after) are given above the major branches (> 90% values).

4. Discussion

4.1. Insight into species concepts and delimitation

The modern age of species concepts began with the use of the term 'concepts' to describe several different approaches to species identification (Mayr, 1942). This resulted in a long list of alternative species concepts (Hey et al., 2003). Indeed, just as there are "a thousand Hamlets in a thousand people's eyes", so it is with the species concepts. Recent decades have also witnessed increasing categories of species concepts and associated debates, including the biological, evolutionary, genetic, phylogenetic species concept, and many others, with Zachos (2016) reporting 32 widely recognised species concepts. Furthermore, species delimitation has been confused by a problem involving the concept of the species itself, namely that the process of speciation is continuous and will thus create inherently fuzzy boundaries, while in practice clear delineation of boundaries is required (Zachos, 2016).

If nature is discontinuous, species delimitation should be possible to identify limits between clusters of organisms once the organisms have been described as thoroughly as possible (Galtier, 2019). Therefore, using *Engelhardia* as a case for integrative approaches to species delimitation, we would expect to obtain a clearer definition of species boundaries. However, it has been argued that scientists should not confuse the detection of species with a theoretical understanding of the way in which species exist (de Queiroz, 1998, 2007). This argument points to the difficulties of studying real species, and asserts that the best understanding of species includes recognition acceptance of their indistinct nature. Consequently, it is a challenge separating detection methods from more basic ideas on the existence of species (Hey et al., 2003).

To prevent confusion and simplify classification, particularly with respect to species delimitation of tropical species, we suggest that the concept of subspecies and varieties should be used cautiously. For example, within *Engelhardia*, *E. serrata* has been attributed four varieties and *E. spicata* three (Manning, 1966). In contrast, our results

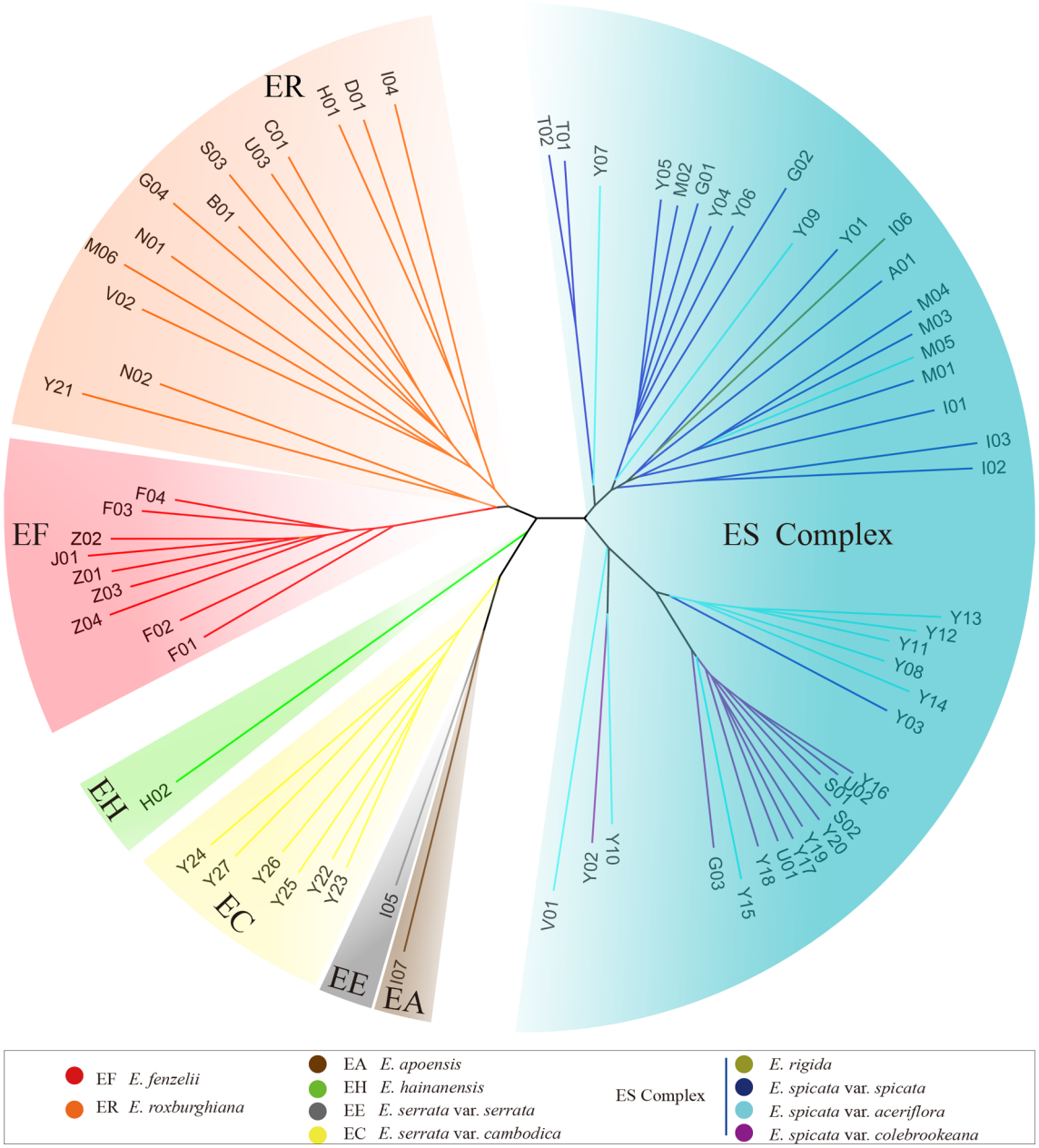

Fig. 4. Phylogenetic analyses of the Neighbor-joining method (NJ) based on nSSR. The colours represent different species as in Fig. 2. The coloured backgrounds enclose individuals belonging to the same cluster.

statistically identified independent or combined lineages, according to a large number of morphological and molecular data analyses. For example, indicating the status of *E. serrata* var. *cambodica* as an independent species. Given that cryptic species are sometimes not distinguishable morphologically due to character convergence, and that more than one species may be present in a group with unclear limits between them. Our results suggest that *E. rigida*, and varieties of *E. spicata*, should be treated instead as a species complex.

In the study of plant speciation, multiple lines of evidence are typically used to re-rank ambiguous morphological variation, but rarely refer to intraspecific classification (Hong, 2010; Hu et al., 2015; Posso-Terranova and Andrés, 2018). Indeed, identifying and analysing genetic clusters is a widely accepted approach, however, whether or not they should be called species, subspecies, or populations is often considered to be an uninteresting secondary issue (Galtier, 2019). The species category is objective, whereas subspecies and varieties are not, and intraspecific classifications do not have ontological status as evolutionary units, rendering them as superfluous evolutionary research (Zachos, 2016). Accordingly, we prefer to recognise only species, or species complexes here.

4.2. Mutual utilization and promotion from morphological and molecular data

The cluster numbers of species depend on arbitrary thresholds or parameters (Posso-Terranova and Andrés, 2018). Research on *Leptolalax* species (Megophryidae) based on extensive geographic and taxonomic sampling showed that molecular data alone could not resolve the number of species (Chen et al., 2018). Based on the hypothetical and simplified divergence tree presented in Fig. 7, different subdivisions of monophyletic clades can easily alter the number of species. For example, the following three taxonomic options all allow for monophyly: (1) populations A–B as one species, and populations C–G as one species, are considered as a two-species option (Fig. 7a); (2) populations A–B,

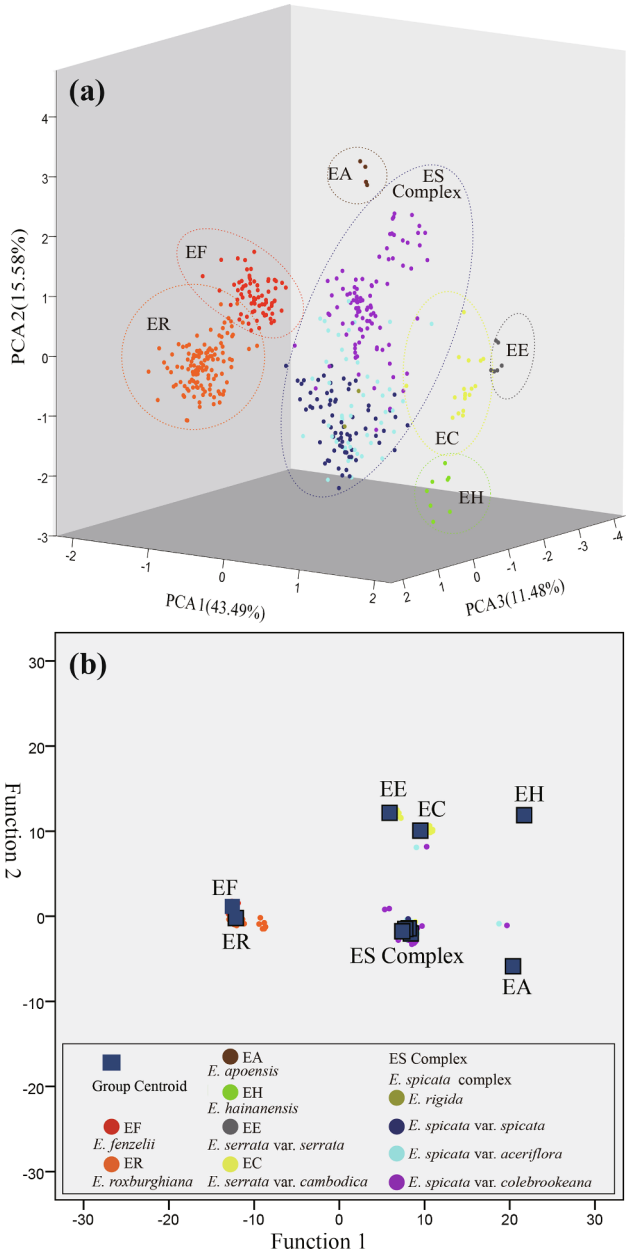

Fig. 5. (a) Scatterplots based on principal component analysis (PCA) scores for each individual (shown as a dot) evaluated. The coloured and dotted circles enclose individuals belonging to the same component. (b) Canonical discriminant analysis. The blue squares represent each group centroid of classification, and the dots represent off-centre individuals. The list of taxa and colour scheme are the same in (a) and (b). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

population C, population D, and populations E–G representing separate species, are considered as a four-species option (Fig. 7b); and (3) if the branches A–G represent one species each, the clades would indicate a seven-species option (Fig. 7c).

Our genetic data analyses reflected the possible relationships within *Engelhardia*. Combining the molecular and morphological characters refined their results by suggesting species boundaries, such as *E. roxburghiana* and *E. fenzelii* separated into two lineages, while *E. spicata* varieties and *E. rigida* combined as a single species (Figs. 3 and 5).

These results have therefore helped to clarify an issue that has plagued species delimitation in *Engelhardia* for many years and we suggest that adopting integrated approaches from multiple-locus DNA and morphological datasets is the most efficient way to delimit the species boundaries, especially in taxa where traditional approaches have not been effective. The advantage of this approach is that the molecular data provide a basic phylogenetic framework for the recognition of lineages, while the addition of morphological data helps to further support the precision and accuracy of systematic and species delimitation.

4.3. Species delimitation in Engelhardia

In this study, the data from the specimens deposited in the herbaria and the large-scale field samples collected throughout the range provide more solid evidence to help resolve the species boundaries in *Engelhardia*.

Engelhardia fenzelii has been considered to be a synonym of *E. roxburghiana* (Lu et al., 1999; Manning, 1966). However, our results revealed that *E. fenzelii* should be recognised as a clearly separate species based on plastid DNA, ITS region, and nSSR data (Figs. 2–4 and 6). Furthermore, structural analysis showed two independent genetic population structures (Fig. S5), and Discriminant Analysis and PCA indicated that their morphological clusters were distinct (Fig. 5). Although both species have similar, terminal inflorescences and glabrous flowers and fruits, *E. fenzelii* possesses greyish white twigs, 1–2 pairs of leaflets, and 4 (3–6) pairs of secondary veins on each leaflet. The twigs of *E. roxburghiana* are dark brown or black, with 3–5 pairs of leaflets and 7 (5–13) pairs of secondary leaflet veins (Fig. S5; see also Keren and Lu, 1979). It is worth noting that herbarium specimens generally do not show detailed information such as colour, possibly contributing to the historical combination of these two species. Additionally, the geographical distribution of these two species does not overlap (Fig. 1): *E. fenzelii* is restricted to eastern China, while *E. roxburghiana* is distributed widely across tropical and subtropical Asia. The classification and phylogenetic relationships within the two species contributed new evidence to distinguish their close affinity, but not their identity (Figs. 2–6). Also, this study contributes new evidence to address the classification of *E. roxburghiana* (*Alfaropsis roxburghiana*). The results do expand on the phylogenetic break within the genus, and recognise two basic clades (Figs. 3, 4 and 6).

Engelhardia rigida shared an identical genetic structure, phylogenetic relationships, network, and morphological characteristics with *E. spicata* var. *spicata* (Figs. 2–6 and S6) and their geographic distribution overlaps (Manning, 1966). Indeed, a previous study indicated that the difference between the two species only includes the intangible lengths of fruiting catkins, the number of stamens, and monoecy vs. dioecy, which suggested that *E. spicata* and *E. rigida* are the same species. In addition, the specimens of *E. rigida* collected from New Guinea were identified as *E. spicata* (Manning, 1966). We found the only apparent differences between samples identified as *E. rigida* and *E. spicata* were the size of leaves, flowers and inflorescences (Fig. S6), and these differences were affected by environmental factors such as rainfall (Dudley, 1996). Therefore, we consider that *E. rigida* should be synonymised with *E. spicata*.

Engelhardia spicata var. *aceriflora* and *E. spicata* var. *colebrookeana* used to be considered independent species (Keren and Lu, 1979), but were subsequently maintained as varieties (e.g., Lu et al., 1999). *E. spicata* and its infrataxa exhibit highly variable, intergrading morphology without any geographical pattern, leading Jacobs (1960) to suggest that the varieties were of no taxonomic value. Similarly, both Discriminant Analysis and PCA showed that the *E. spicata* varieties are not supported by morphology (Fig. 5). The species occupies a broad range of habitats and the variation in leaflet morphology reflects this. *E. spicata* var. *spicata* is a part of evergreen forest tree communities in hilly regions, but during our fieldwork in Indonesia, we found specimens with entire adult leaflets but serrated juvenile ones, similar to those of *E. serrata* (Fig. S8). In addition, more hirsute leaflets of *E. spicata* var. *colebrookeana* seem to be associated with steep dry slopes on sandy

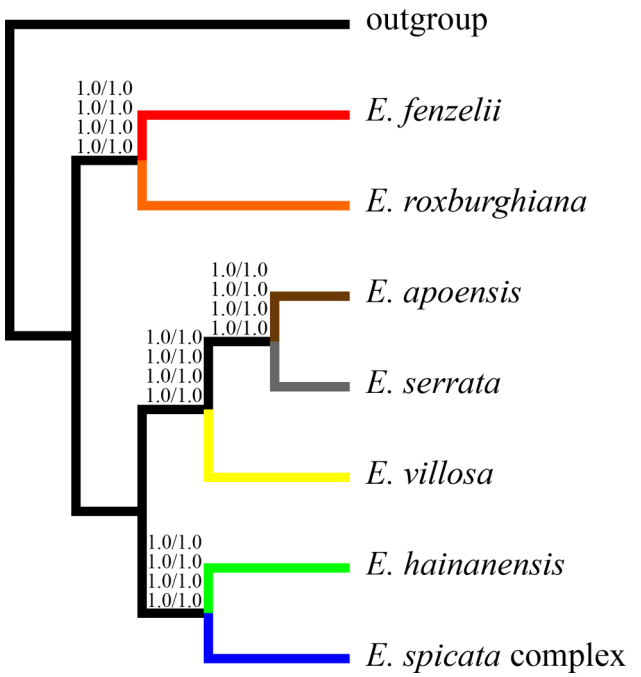

Fig. 6. Schematic of the species delimitation hypotheses inferred. The BPP analyses used molecular data and the iBPP analyses used combined molecular and morphological data sets. Numbers at each node of the tree represent the posterior probability for that node inferred by BPP (before) and iBPP analyses (after). The numbers from upper to lower correspond to analyses with the four different priors: θ = G(1, 10) and τ = G(1, 10); θ = G(1, 10) and τ = G(2, 2000); θ = G (2, 2000) and τ = G(1, 10); θ = G(2, 2000) and τ = G(2, 2000). The *E. spicata* complex includes *E. spicata* var. *spicata*, *E. spicata* var. *aceriflora*, *E. spicata* var. *colebrookeana*, and *E. rigida*.

soils, whereas the thick leathery leaflets of *E. spicata* var. *aceriflora* limit the evaporation of water. However, regardless of leaflet morphology, their terminal buds, inflorescences, and fruit are identical. Indeed, the present phylogenetic analyses and species tree inference suggested that all evolved recently (Figs. 3–4 and 6); and the cpDNA and nrDNA data highlight their complex genetic network (Fig. 2). The genetic structure indicates that some *E. spicata* var. *aceriflora* populations are related to either *E. spicata* var. *colebrookeana* or *E. spicata* var. *spicata* (Fig. S6). This morphological complexity seems to indicate repeated divergences with incomplete reproductive isolation and high levels of reticulate interspecific gene flow. As non-monophyly may result from hybridization, incomplete lineage sorting and/or insufficient genome sampling, we recommend that *E. spicata* be treated as a species complex that includes *E. spicata* var. *aceriflora*, *E. spicata* var. *colebrookeana*, and *E. rigida*.

Engelhardia hainanensis, *E. apoensis*, *E. serrata* var. *serrata*, and *E. serrata* var. *cambodica* all represent clear, separate evolutionary lineages (Figs. 2–6, Fig. S7), although *E. apoensis* was sister to *E. serrata* in the phylogenetic trees, their morphology is completely different. The leaflets of *E. apoensis* are large, thick, entire, lightly hirsute, oblique at the base, and similar in size on the same branch, whereas *E. serrata* leaflets are subsessile, serrate, decrease in size distally, and have a lightly hirsute rachis (Lu et al., 1999; Manning, 1966). Our field observations of *E. apoensis* further identified additional unique characteristics, including convex scales on the leaflet surface (Figs. S1 and S7). The placement of *E. apoensis* with *E. serrata* in the analyses might reflect historical gene flow between geographically close populations, or small sample size reducing the accuracy of the phylogeny (Edwards and Knowles, 2014). Small sample size is a problem for rare species with narrow distributions (Federman et al., 2018), a distinct possibly since we only found a single population of *E. apoensis* with two trees, and a total of 14 trees of *E. serrata* in our field survey. Actually, low sample sizes have always restricted understanding of their gene phylogeny, but increasing in the number of samples may support more solid species delimitation. In this study, it is difficult to determine whether each clade represents one species in the phylogenetic trees, which are based on few samples (Fig. 7c). However, better subdivisions of monophyletic clades will increase the accuracy of species delimitation when additional samples are (Fig. 7a). *E. hainanensis* is endemic to Hainan Island and shows well-differentiated morphology (Fig. 5), phylogenetic clustering (Figs. 2–4), and Bayesian species delimitation (Fig. 6). Structural analysis (Fig. S7) assisted in separating the evolutionary lineage from other *Engelhardia*. There is also supportive evidence for *E. serrata* var. *cambodica*, including a monophyletic group in the phylogenetic tree (Figs. 2–4, 6) and statistically significant differences in morphologies (Fig. 5), suggesting that this variety should be reranked as a species and resumed *E. villosa* Kurz (Kurz, 1877). *E. villosa* was treated as a synonym of *E. serrata* var. *cambodica* (Manning, 1966). According to the *International Code of Nomenclature for algae, fungi, and plants* (Turland et al., 2018), and the results from comprehensive analyses in this study, *E. villosa* should be resumed an independent species.

In summary, the genus *Engelhardia* contains seven genetically and morphologically supported species, i.e., *E. roxburghiana*, *E. fenzelii*, *E. apoensis*, the *E. spicata* complex, *E. hainanensis*, *E. serrata*, and *E. villosa*. A key to the species of *Engelhardia* is provided after the Conclusions section.

5. Conclusions

In recent years, modern methods for species delimitation have provided biologists with an increased ability to assess diversity more accurately. However, species delimitation still remains a challenge worldwide, especially in biodiversity hotspots such as tropical and subtropical Asia. An integrative method based on multiple-locus genetic data and morphological analyses was used to delimit seven species within *Engelhardia*. Four species (*E. hainanensis*, *E. apoensis*, *E. serrata*, and *E. roxburghiana*) retain their current taxonomic status. *E. fenzelii* is resurrected from *E. roxburghiana*, and *E. spicata* is expanded to become a variable species complex to include *E. spicata* var. *aceriflora*, *E. spicata*

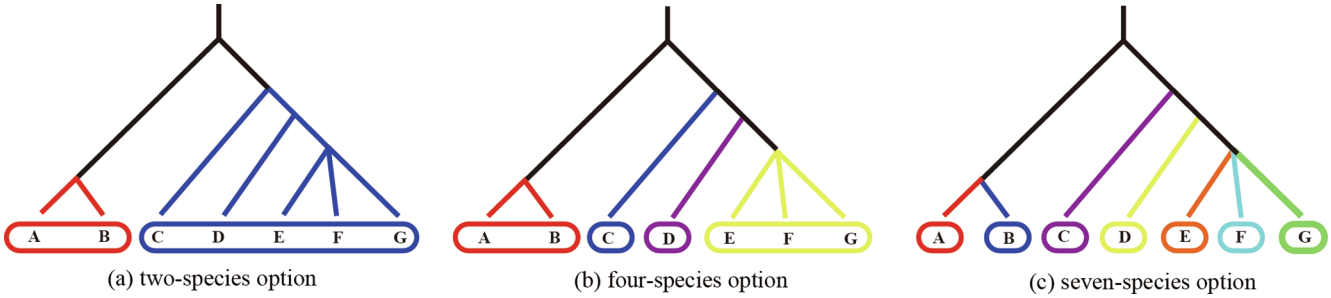

Fig. 7. A hypothetical and simplified flow chart for phylogenetic analyses based on DNA haplotype. A–G are operational taxonomic units. Three taxonomic options allow for monophyly: (a) denotes a two-species option; (b) denotes a four-species option; (c) denotes a seven-species option.

var. *colebrookeana*, and *E. rigida*. Finally, *E. serrata* var. *cambodica* is re-ranked as an independent species. This study further highlights the importance of mutual utilization and promotion of morphological and molecular data. That is, morphological statistics can be used to solve the problem of defining criteria suitable for evaluation within a phylogenetic framework without defined lineages. Also, our study suggests that the recognition of infraspecific taxa should be done with caution in order to simplify classifications and prevent confusion. Specifically, re-ranking or combining subspecies and/or varieties may, in some cases, enable more accurate species delimitation.

Key to the species of *Engelhardia*

1. Inflorescences terminal; pistillate flowers and fruits glabrous, prominently stalked; bracts at fruit base; terminal bud glabrous, comb-like; leaves evergreen; leaflets entire, glabrous, conspicuously stalked.
2. Twigs dark brown or black; leaflets usually 3–5 pairs, the majority shortly acuminate at apex, the secondary leaflet veins 7(5–13) pairs. ……..
……***E. roxburghiana***
2. Twigs grayish white; leaflets only 1–2 pairs; the majority acuminate at apex; the secondary leaflet veins 4 (3–6) pairs.
……***E. fenzelii***
1. Inflorescences lateral; pistillate flowers and at least the base of fruit hairy, typically subsessile; bracts cover the fruit; terminal bud hirsute; leaves evergreen or deciduous; leaflets serrate or entire, glabrous or hirsute, stalked or sessile.
3. Leaflets entire, lightly hirsute, elliptic at apex, oblique at base; similar leaflet size on same branch……..……***E. apoensis***
3. Leaflets entire or serrate, glabrous or hirsute, acuminate or elliptic at apex, rounded or oblique at base; the lower leaflets reduced in size or gradually becoming smaller.
4. Leaflets usually entire or serrate just in the sapling, somewhat variable in size and shape, glabrous to densely hirsute, acuminate or elliptic at apex, usually the lower leaflets reduced in size. ……..……***E. spicata* complex**
4. Leaflets serrate, glabrous or hirsute, acuminate at apex, leaflets gradually becoming smaller or lower leaflets strongly reduced in size.
5. Leaflets sessile, glabrous to slightly pubescent along midvein abaxially; lower leaflets strongly reduced in size; branchlet glabrous..
……***E. hainanensis***
5. Leaflets sessile or subsessile; glabrous or hirsute; leaflets gradually becoming smaller; branchlet hirsute.
6. Leaflets sessile, glabrous, branchlet lightly hirsute; the secondary leaflet veins 7-(6–10) pairs.
……***E. serrata***
6. Leaflets sessile or subsessile, densely hirsute, branchlet densely hirsute; the secondary leaflet veins 6 (5–10) pairs……..
E. villosa

CRediT authorship contribution statement

Can-Yu Zhang: Conceptualization, Investigation, Writing - original draft, Writing - review & editing, Visualization. **Shook Ling Low:** Investigation, Writing - review & editing. **Yi-Gang Song:** Writing - review & editing. **Nurainas:** Investigation, Resources. **Gregor Kozlowski:** Writing - review & editing. **Lang Li:** Investigation, Resources. **Shi-Shun Zhou:** Investigation, Resources. **Yun-Hong Tan:** Writing - review & editing. **Guan-Long Cao:** Investigation, Resources. **Zhuo Zhou:** Investigation, Resources. **Hong-Hu Meng:** Conceptualization, Investigation, Writing - original draft, Writing - review & editing, Supervision. **Jie Li:** Conceptualization, Writing - review & editing, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

Many thanks to the following people for their kind help in this study: Dr John Conran for English language revision, Ismail, Dasriel Effendi, and Harry for assistance in the field, Dr Richard Chung-Cheng Kong for access to digital herbarium specimens, and Xiao-Long Jiang, Bin Tian, Jian-Li Zhao, Li Li, Wu-Mei Xu, Yong-Ling Yin, Xiu-Yan Feng, Hang-Hui Kong, Han-Yang Lin, Jian-Hua Xiao, Chao-Nan Cai, Zhi-Fang Liu, and Jian-Lin Hu for assistance with data collection and analysis. This work was supported by the National Natural Science Foundation of China (No. 41701056); Yunnan Fundamental Research Projects (No. 2018FB032); Southeast Asia Biodiversity Research Institute, Chinese Academy of Sciences (No. Y4ZK111B01); Youth Innovation Promotion Association, Chinese Academy of Sciences (No. 2018432); the CAS "Light of West China" Program; and the Program of the Chinese Academy of Sciences (No. 2017XTBG-T03).

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ympev.2020.106918.

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