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RESEARCH ARTICLE

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Evolution of woody life form on tropical mountains in the tribe Spermacoceae (Rubiaceae)[1]

Suman Neupane[2,6], Paul O. Lewis[2], Steven Dessein[3], Hunter Shanks[4], Sushil Paudyal[4], and Frederic Lens[5]

PREMISE OF THE STUDY: Spermacoceae are mainly an herbaceous group in the Rubiaceae. However, a few lineages are woody and are found in a diverse range of habitat types. Three of the largest woody lineages (*Arcytophyllum*, *Hedyotis*, and *Kadua*) are characterized by their distribution in the moist tropical mountains and have disjunct distribution patterns with respect to their closest relatives. In this study, we explore the cases of derived woodiness in these three lineages and their diversification dynamics in the tropical mountains of Asia, the Pacific, and the Americas.

METHODS: By combining phylogenetic results with wood anatomical studies, we estimated timing of origin of the three woody groups, inferred their ancestral traits and ancestral distribution ranges, analyzed their associations with the tropical upland habitat, and elucidated their diversification across tropical mountains.

KEY RESULTS: The three woody clades originated and diversified from herbaceous ancestors in close association with the tropical upland habitat during the Miocene. The ancestral range for Asian-Pacific *Hedyotis* is Africa/Madagascar and continental Asia for Pacific *Kadua*. The complex geological history of tropical Asia allowed *Hedyotis* to diversify faster and create narrow endemics near oceans in the highlands of the Western Ghats in India, Sri Lanka, Southeast Asia including southeastern China, and New Guinea.

CONCLUSIONS: The three major woody clades in Spermacoceae have gained their woodiness independently from one another, subsequent to colonization by their ancestors from a different geographic environment. The evolution and diversification along the tropical mountain orogeny is strongly linked with the formation of woody habit and many narrow endemic species.

KEY WORDS diversification; Rubiaceae; secondary woodiness; Spermacoceae; tropical mountains; wood anatomy

Angiosperms, in contrast to the woody gymnosperms, are characterized by a huge diversity in woody and herbaceous life forms (Rowe and Paul-Victor, 2012). Since angiosperms developed from woody gymnosperm-like ancestors, the woody state is believed to be ancestral within angiosperms, meaning that all herbaceous angiosperms have lost wood formation during their evolution, although it is known that most herbaceous, nonmonocot angiosperms still produce a limited amount of wood at the base of their stems (Schweingruber et al., 2011; Lens et al., 2012a, b; see also "hidden wood formation" sensu Rowe and Paul-Victor, 2012). Remarkably, many of these herbaceous lineages have regained the woody habit and have developed into woody shrubs and even trees on islands or tropical mountain peaks (Darwin, 1859; Carlquist, 1974). This phenomenon is known as derived or "secondary" woodiness. The evolutionary reversal back to the woody ancestral state has occurred multiple times independently (e.g., Lens et al., 2013a), but we still do not have sound experimental evidence for how wood formation is triggered and whether environmental cues initiating wood are taxon-dependent.

Spermacoceae are mainly a pantropical tribe (~1000 species, 60 genera, Govaerts et al., 2016) in the family Rubiaceae. Recent molecular phylogenetic studies (Kårehed et al., 2008; Groeninckx et al., 2009; Guo et al., 2013; Wikström et al., 2013; Neupane et al., 2015) have helped resolve many taxonomic inconsistencies within the group. The tribe is dominated by herbaceous species that are often weedy in nature (Groeninckx et al., 2009), but there are also woody

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species, ranging from (sub)shrubs to climbers, to even understory trees 7 m tall [e.g., *Nesohedyotis arborea* (Roxb.) Bremek.; Percy and Cronk, 1997; see Table 1; Lens et al., 2009]. The woody Spermacoceae species occur in a wide range of environmental conditions, including markedly dry areas in southwestern Madagascar; mist-covered areas of tropical montane cloud forests in Asia, South America, and Africa; and tropical islands across the Pacific (Table 1). Lens et al. (2009) suggested that woodiness within Spermacoceae evolved at least 10 times and is secondarily derived from an herbaceous condition. These transitions toward the woody life form have been inferred by molecular phylogenies, but the authors also provided additional support by showing the presence of typical primary xylem characters that are retained in the wood of the few woody species observed. Carlquist (2009, 2012) referred to this wood anatomical pattern as "protracted juvenilism". Examples of these juvenile wood characters are rays with mainly upright cells or total absence of rays, (pseudo-)scalariform intervessel pitting, and flat or decreasing length-on-age plots for vessel elements (Carlquist, 1962, 2001, 2009; Lens et al., 2009, 2012a, 2013a; Dulin and Kirchoff, 2010).

TABLE 1. Summary of habit, habitat and distribution of the woody clades in the tribe Spermacoceae compiled from references in Appendix S2.

Taxon	Habit	Habitat and distribution
Amphistemon humbertii	Branched subshrub up to 0.5 m	Dry spiny forest–thicket in xerophyllous scrubland and on calcareous substrates in Madagascar at 2–740 m a.s.l.
Arcytophyllum	Erect or prostrate perennial subshrubs or shrubs up to 1.7 m	High mountains of Costa Rica and Panama, wet paramos and subparamos (few also in pumas) of northern Andes at 400–4250 m a.s.l.
Bouvardia	Shrubs or perennial herbs up to 2.5 m	Rocky outcrop of steep hillsides or barrancas in Mexico to northern Central America above 500 m a.s.l.
Dimetia	Perennial lianascent climber	Mountain slopes, ravines and valleys of (sub)tropical Asia at 200–1800 m a.s.l.
Emmeorhiza umbellata	Liana	Disturbed areas, forests, riversides, shrublands at 0–2500 m a.s.l.
Ernodea	Erect or prostrate shrub	Mostly endemic to Bahamian Archipelago in coastal areas in sand dunes, near mangrove communities, limestone, disturbed open areas, or pinelands in southern Florida
Galianthe brasiliensis	Shrub up to 1 m	Southern Mexico to tropical South America in varied habitat from disturbed areas, riparian forest at 1350–1700 m a.s.l.
Galianthe chiquitosiana	Shrub up to 1 m	On rocky slopes and fields of Bolivia (Santa Cruz)
Hedyotis	Very few herbaceous, some suffrutescent herbs, mostly shrubs or small trees up to 7 m	Mid or high elevation slopes (to 4000 m a.s.l.) of Asia, Papuasia, and NW Pacific, some species <200 m a.s.l.
Hedythyrsus spermacocinus	Shrub or subshrub up to 1.2 m	Source of river Zambezi, Mushitu fringes and dambo/mushitu edges, Zambia at 1100 m a.s.l.
Hedythyrsus thamnoideus	Subshrub up to 2 m	Malawi North Nyika Plateau, Zambia and North Mafinga mountains and Uluguru mountains in Tanzania at 1450–2880 m a.s.l.
Kadua	Scandent shrubs or lianas to small or medium sized trees up to 6 m	Lower to mid elevation slopes in Hawaiian Islands, and French Polynesia at 60–2438 m a.s.l.
Lathraeocarpa acicularis	(Sub)shrubs, with woody stems and well-developed woody taproots up to 25 cm	Sandy soils in dunes close to the sea in Madagascar
Mitracarpus anthospermoides	Small shrub	Endemic to Brazil, in restinga habitat (coastal (sub)tropical forest on white sand) between Salvador and Camaçari
Mitracarpus frigidus	Subshrub up to 1 m	Scrubland, grassland, and in disturbed areas at 150–2970 m a.s.l.
Mitracarpus portoricensis	Shrub up to 0.6 m	Sandy plains and hillsides in moist parts of the northern and western districts of Puerto Rico
Mitracarpus rigidifolius	Shrub up to 1 m	Endemic to Bahia state (Brazil), between Morro do Chapéu, Umburanas, Xique-Xique and Gentio do Ouro on sandy soils at 500–1100 m a.s.l.
Mitracarpus robustus	Shrub up to 2 m	From Brazil to French Guiana at 40–1350 m a.s.l.
Nesohedyotis arborea	Tree up to 7 m	Upper ridge among moist fern thickets in St Helena island above 700 m a.s.l.
Pseudonesohedyotis	Shrublet or liana	Mist forest in Uluguru mountains in Tanzania at 1370–2650 m a.s.l.
Psyllocarpus	Shrub up to 1.2 m	Savannas in Planalto Central do Brasil and white-sands vegetation in Amazon valley
Scandentia cymosa	Climbing shrub	In Atlantic forest of Brazil from Alagoas State to Rio de Janeiro State
Scandentia monodon	Climbing shrub	In Atlantic forest of Bahia State (Brazil)
Spermacoce bangweolensis	Prostrate or suberect subshrub with woody taproot up to 50 cm	Around Lake Bangweulu, Zambia at 1174 m a.s.l.
Spermacoce manikensis	Geoxylic perennial herb with woody tap root	Dry areas of Manika high plateau, Katanga, DR Congo at 1500 m a.s.l.
Spermacoce bangweolensis	Small shrub up to 0.5 m	On bare soil among grass clumps at Lake Bangweulu (Samfya, northern Zambia) at 1150 m a.s.l.
Spermacoce brachyantha	Small shrub	Windblown sand over limestone in low open Acacia-Commiphora bushland in Somalia
Spermacoce dispersa	Shrub up to 0.6 m	In sandy, rocky, or tufaceous soil and on lava flows on Galapagos Islands
Spermacoce linearifolia	Shrub up to 0.5 m	On tufaceous soils in openings in vegetative cover, among rocks, or in crevices on lava flows (Galapagos Islands)
Spermacoce resinosula	Shrub	On top of vegetated sand hills in Tanami Desert east of Western Australia border (Northern Territory, Australia)
Spermacoce verticillata	Shrub	Vegetated sand hills in the Tanami Desert (Northern Territory, Australia)
Thamnoldenlandia ambovombensis	Brached medium sized shrub up to 1.5 m	Southwestern dry spiny forest–thicket, in Madagascar at 150–190 m a.s.l.

In this paper, we build on the results of Lens and coworkers (2009) and combine more wood anatomical observations with up-to-date phylogenetic analyses to provide sound support for the secondary origin of woodiness in Spermacoceae, investigate biogeographical patterns, and assess whether woodiness is correlated with increased speciation within the tribe. To do so, we focus on the three largest woody clades in Spermacoceae, i.e., *Arcytophyllum* (16 species), *Hedyotis* (ca. 180 species), and *Kadua* (29 species), which consist of predominantly woody shrubs or small trees (Table 1). Interestingly, the three genera show a disjunct pattern of distribution with respect to their closest relatives. For example, *Arcytophyllum* is mainly distributed across the highlands of the Andes (Mena, 1990), but their closest (herbaceous) relatives (Andersson et al., 2002; Kårehed et al., 2008; Groeninckx et al., 2009) are the North American *Houstonia* (23 species)/*Stenaria* (6 species), and *Stenotis* (7 species) group (Terrell, 1996, 2001a, 2001b; Govaerts et al., 2016). *Hedyotis* (Neupane et al., 2015) is a predominately tropical montane group distributed in Asia and the Micronesian Islands (northwestern Pacific), but its sister group *Agathisanthemum-Edrastima* comprises African/Malagasy elements (*Agathisanthemum* and *Lelya*, 5 species, Govaerts et al., 2016), and an African–tropical Asian–North American group (*Edrastima*, 5 species, Neupane et al., 2015). Similarly, the Polynesian *Kadua* is mainly distributed along the wet mid-elevation zones of the Hawaiian Islands (22 species) and on several islands of the southern-central (7 species) and southwestern Pacific (1 species) (Wagner and Lorence, 2002; Wagner et al., 2005; Kårehed et al., 2008; Groeninckx et al., 2009; Parker, 2012; Govaerts et al., 2016), while its closest relative, *Leptopetalum* (8 species), is native to the lowlands of Asia and the Pacific (Fosberg and Sachet, 1991; Wikström et al., 2013; Neupane et al., 2015). In addition to the disjunct biogeographic patterns, the difference in the number of species in *Hedyotis* and *Kadua*, with respect to their closest relatives, is disproportionately larger.

These interesting biogeographic patterns between closely related lineages with disproportionate species diversity and contrasting herbaceous–woody life forms in different habitats make the pantropical Spermacoceae an ideal study system in which to investigate the tempo and mode of ancient dispersal patterns into a range of habitats throughout the world and also to study its effect on lineage diversification and life form shifts toward secondary woodiness. Therefore, building on the existing data sets (Kårehed et al., 2008; Groeninckx et al., 2009; Guo et al., 2013; Wikström et al., 2013; Neupane et al., 2015), and adding more samples (59 accessions, 178 sequences) from the Pacific and North America, we performed a phylogeny-based biogeographic, trait evolution, and lineage diversification study, combined with data from geological history and wood anatomical observations, to address the following questions: (1) When did the three woody groups originate, and from where did their ancestors come? (2) What are the ancestral states of habit and habitat types of these groups? (3) Is there any association between the tropical upland habitat and secondary woodiness? (4) What is the effect of this potential link in the diversification of the woody clades compared with their herbaceous relatives? (5) What is the role of ancient biogeographic events in the current distribution of the woody clades?

MATERIALS AND METHODS

Taxon sampling, DNA extraction, alignment, and chronogram estimation—To make inferences about biogeographic history, lineage diversification, and trait evolution, we performed a time-calibrated Bayesian phylogenetic analysis of Rubiaceae and Spermacoceae. Multiple sets of data were used for this purpose (Table 2). First, we studied divergence time estimates of the three woody clades (*Arcytophyllum*, *Hedyotis*, and *Kadua*) within Spermacoceae utilizing fossil calibration from outside the tribe. Recently, Wikström et al. (2015) estimated the time tree of asterids (114 taxa) and Gentianales (72 taxa) by expanding on the data from the previous studies (Bremer et al., 2004; Bremer and Eriksson, 2009). Therefore, DNA sequences representing all tribes in the Rubiaceae were extracted from the study by Wikström et al. (2015). This Rubiaceae data set was expanded by including more sequences of the Spermacoceae (Appendix S1, see Supplemental Data with this article), which represented both the woody and herbaceous clades (total 110 species), to obtain age estimates of the major clades within Spermacoceae. However, our data matrix only included four (*rps16*, *trnL-trnF*, *matK*, and *rbcL*) of the six plastid markers used by Wikström et al. (2015) due to the lack of *trnV* and *ndhF* sequences for Spermacoceae. A four-gene (*rps16*, *trnL-trnF*, *matK*, and *rbcL*) chronogram was obtained by placing four fossil constraints: (1) *Scyphiphora* sp. (Rubiaceae: Ixoroideae), Early Miocene, 16 Ma; (2) *Cephalanthus pusillus* (Rubiaceae: Cinchonoideae), Late Eocene, 34 Ma; (3) *Morinda chinensis* (Rubiaceae: Rubioideae), Late Eocene, 38 Ma; (4) *Alstonia* R.Br. (representing stem age of Apocynaceae) Middle Eocene, 47 Ma. Additionally, a normally distributed secondary root calibration (Gentianales crown age) with mean 75 Ma and standard deviation of 7.7 was enforced as done by Wikström et al. (2015).

Secondly, by expanding on previous work (Kårehed et al., 2008, Groeninckx et al., 2009; Wikström et al., 2013; Neupane et al., 2015) and adding our newly generated sequences (59 accessions, 178 sequences), a separate five-gene (ITS, ETS, *petD*, *rps16*, and *trnL-trnF*) chronogram of Spermacoceae was estimated by constraining a normally distributed root-height (obtained from the earlier Rubiaceae chronogram) for other comparative studies (trait evolution, lineage diversification, and historical biogeography). In all, 362 terminals (Appendix S1) were chosen for this study. The primers (for ITS, ETS, *petD*, *rps16*, and *trnL-trnF* regions), and the DNA sequencing protocols (DNA extraction, PCR amplification, and sequencing) for the newly sampled Spermacoceae members are described by Kårehed et al. (2008), Groeninckx et al. (2009), and Neupane et al. (2015). DNA sequences were aligned using PRANK (Löytynoja and Goldman, 2008), a phylogeny-aware multiple sequence aligner, through its web interface (available at http://wasabiapp.org). The data sets were partitioned by genes for both analyses (Rubiaceae and Spermacoceae chronograms). The divergence times of different lineages were estimated using molecular dating techniques assuming a relaxed molecular clock model under the Bayesian framework implemented in the program BEAST v1.8.0 (Drummond et al., 2012a). Instead of selecting a particular relaxed clock model for rates of substitution across branches, a model averaging approach (Li and Drummond, 2011) was used,

TABLE 2. Number of accessions and DNA regions used for Rubiaceae and Spermacoceae chronogram.

Phylogeny	No. of accessions	DNA regions with number of accessions used						
		ITS	ETS	*petD*	*rps16*	*trnL-trnF*	*matK*	*rbcL*
Rubiaceae	110	—	—	—	105	96	77	86
Spermacoceae	362	342	251	215	287	135	—	—

as done by Wikström et al. (2015). Two forms of uncorrelated relaxed clock models were specified for this purpose: (1) lognormal relaxed clock (ucld) with exponential prior (ucld.mean = 1.0, ucld.stdev = 0.33), and (2) exponential relaxed clock (uced) with exponential prior (uced.mean = 0.01). The fossil ages selected were used as minimum age constraints tested under exponential and uniform (upper bound = 128 Ma) prior distributions for Gentianales. At least five runs of 100,000,000 generations were performed and checked for convergence using the program Tracer v.1.6.0 (Rambaut et al., 2014). BEAST analyses were conducted using the CIPRES portal (Miller et al., 2010) and the bioinformatics facility cluster at the University of Connecticut, Storrs.

Ancestral character state estimation, trait correlations, and trait-dependent diversification analyses—Chronograms obtained from the Spermacoceae BEAST analysis were chosen for ancestral state estimation for life growth forms and habitat types. Although some clades consist of exclusively annual herbs (e.g., *Debia*, *Edrastima*, *Involucrella*, *Neanotis*, and *Oldenlandia*), many other herbaceous clades (e.g., *Exallage*, *Houstonia*, *Kohautia*, and *Spermacoce*) include members with both annual and perennial herbs that develop a small wood cylinder confined to the base of their stems. As with many dicot taxa (Dulin and Kirchoff, 2010; Schweingruber et al., 2011; Lens et al., 2012a), continuous variation in the degree of wood development between species often makes the boundary between herbaceousness and woodiness fuzzy. As we were only interested in understanding the dramatic shifts toward increased wood formation, we categorized as "woody species" any shrub or tree that develops a wood cylinder that extends toward the upper parts of the stems, thereby discarding all the intermediate life forms such as "woody" herbs or suffrutescent perennial herbs (see Lens et al., 2012a, 2013a). Thus, we could characterize the habit within Spermacoceae into two distinct categories: (1) annual and perennial herbs and (2) woody species including climbers, shrubs, and trees (online Appendix S2). For ancestral habitat types, a distinction was made between the species that are found in (1) lower elevation or with widespread distribution and (2) mountain ranges (Appendix S2). While some upland species may be restricted to one of the stratified zones of mountains, others are widespread along the mountain range. Also, some smaller mountains do not have extreme tundra zones (e.g., oceanic island mountains). Therefore, we categorized all high elevational species as "upland" to include any species occurring on the mountains >1000 m a.s.l. Additionally, the evolutionary correlation between habit and habitat forms was tested to assess the role of the tropical upland habitat in woody habit formation by using binary states for each of the traits where habit was distinguished as "woody" vs. "herbaceous", and habitat as "tropical upland" vs. "other". The "other" category included all other habitat forms (e.g., temperate upland, lowland, or mixed habitat of widespread distribution) that were not range-restricted in the wet tropical mountains. The ancestral states were estimated using a Bayesian Markov chain Monte Carlo (MCMC) approach under the discrete MultiState (Pagel et al., 2004) model. The correlation study was performed using discrete independent and discrete dependent models (Pagel and Meade, 2006) where the former (discrete independent) model allows the two traits to evolve independently and the latter model (discrete dependent) assumes the traits to be correlated and the rate of change in one trait to be dependent on the state of the other. These models are implemented in the BayesTraits V2.0 (available from http://www.evolution.reading.ac.uk/BayesTraits.html). For both studies (ancestral state estimation and correlated trait evaluation), a reversible-jump (RJ) MCMC approach was used with a hierarchical model in which a Uniform (0,100) hyperprior was used for the mean of the exponential prior assigned to each rate parameter ("RJHP exp 0 100" command). The RJHP option takes into account the uncertainty in the phylogenetic tree as well as the uncertainty in the estimate of ancestral state and model parameters (Pagel et al., 2004; Pagel and Meade, 2006) by sampling over the Bayesian posterior sample of trees using the MCMC method of character state estimation. A set of 10,000 post burn-in Bayesian chronograms from the Spermacoceae BEAST analysis was used for this purpose, and the posterior probability of each state was mapped on the maximum-clade credibility tree for ancestral state estimation. The discrete independent and dependent models were evaluated from their marginal likelihood values (Bayes factor comparison), which were estimated using the stepping-stone method ("stones 100 10000" command) option available in the package.

We performed a trait-dependent diversification method, HiSSE (hidden state speciation and extinction; Beaulieu and O'Meara, 2016), to examine whether the transitions between binary states of particular traits impacted speciation and extinction rates, thus affecting the overall diversification process within Spermacoceae. The HiSSE method, like the earlier BiSSE model (binary state speciation and extinction; Maddison et al., 2007), includes joint models of trait-dependent speciation, extinction, and transition rates in its likelihood framework. However, HiSSE also allows the models with unknown hidden traits (unobserved) to affect the diversification rate, providing a more realistic scenario of diversification heterogeneity. Allowing codistribution of focal (observed) and hidden (unobserved) traits in the diversification process addresses the concern raised by Rabosky and Goldberg (2015) on the BiSSE model, which almost always supported trait-dependent diversification even if there is none (type I error). We tested 24 diversification models on both habit and habitat traits (Tables 4 and 5; see Beaulieu and O'Meara, 2016 for details of each model), with and without constraints on different diversification parameters (speciation, λ; extinction, μ; and transition rates, q). Four of these models (Model 1–Model 4) correspond to BiSSE, whereas the other 16 (Model 9–Model 24) correspond to HiSSE models that assumed a hidden trait state associated with the observed states. The remaining four models assumed character independent diversification scenarios (CID; Model 5–Model 8). The clade-specific sampling frequency was assigned to account for incomplete sampling in the phylogeny. All models were implemented in the R package hisse (Beaulieu and O'Meara, 2016), where the model fit was assessed using the Akaike information criterion (AIC). The HiSSE analysis was performed on the Spermacoceae maximum clade credibility tree.

Ancestral range estimation analysis—Different models of geographic range evolution were considered to infer historical biogeographic events within Spermacoceae. For this purpose, we used the R package BioGeoBEARS (Matzke, 2013a), which analyzes biogeographic scenarios under various models in a common likelihood framework. The models implemented in this package are DIVA (Ronquist, 1997) and BAYAREA (Landis et al., 2013) in its likelihood forms (as DIVA-like and BAYAREA-like), and DEC (Ree et al., 2005) with or without an extra founder-event speciation process parameter (+J, Matzke, 2013b). Additionally, this package

also allows one to choose the best among various models using model choice procedures such as the likelihood ratio test (LRT) and the AIC. The biogeographic areas were defined based on the current distribution of taxa and the geological history of their extant ranges. The distributional range of each species was obtained from the "World Checklist of Rubiaceae" (Govaerts et al., 2016) and other regional and local floras (see references in Appendix S2). To understand the origin and ancestral range of *Hedyotis* within the *Hedyotis-Agathisanthemum-Kohautia* clade, seven biogeographic areas were defined: North America (A), Africa including Madagascar (B), Sri-Lanka and Indian western Ghats mountains (C), Southeast Asia west of Wallace's line including Southeast China and the Philippines (D), Sundaland (E), east of Wallace's line comprising Melanesia and tropical part of Australia (F), and Micronesia (G). For *Kadua* and the related group (Asian-Pacific group, see Neupane et al., 2015), six biogeographic areas were specified: Continental Asia (A), Australia (B), Northwestern Pacific (C), Southwestern Pacific (D), North-Central Pacific (E; Hawaiian Islands), and South-Central Pacific (F; French Polynesia) following the demarcation in the "World Geographical Scheme for Recording Plant Distributions" by Brummitt et al. (2001). To avoid the possibility of a speies simultaneously occupying implausible areas (areas geographically isolated by large ocean gaps) and affecting subsequent likelihood estimations, the areas adjacency option was activated by assigning the adjacency matrix (Appendix S3). The BioGeoBEARS analysis was performed on the Spermacoceae maximum clade credibility tree.

Wood anatomical study—To provide further support for the signature of secondary woodiness from the anatomical point of view (cf. Lens et al., 2009), we increased sampling using mature wood samples (online Appendix S4) in the most diverse woody clade (*Hedyotis*, 23 samples), a woody climber group (*Dimetia*, 2 samples), and perennial herbs that maintain some degree of woodiness at the base (*Exallage*, 2 samples). *Hedyotis kurzii* Merr. (diameter of the mature sample: 1 mm), one of the few herbaceous species within the genus *Hedyotis* (Neupane et al., 2015), was investigated and compared with other perennial herbaceous taxa [*Exallage auricularia* (L.) Bremek.] in terms of the degree of wood formation. Transverse, radial, and tangential sections were prepared using a sliding microtome. The subsequent steps after sectioning followed Lens et al. (2007). For the herbaceous samples, only transverse sections were made following the LR White method described by Hamann et al. (2011). The wood anatomical terminology follows the International Association of Wood Anatomists (IAWA) list of microscopic features for hard wood identification (IAWA Committee, 1989) and Lens et al. (2009). The length of vessel elements was measured on radial sections from pith toward cambium to generate the length-on-age curves. Other anatomical features related to protracted juvenilism were recorded (Appendix S4).

RESULTS

Divergence time estimation of Rubiaceae and phylogeny of Spermacoceae—The phylogenetic tree from the combined four plastid genes (*rps16*, *trnL-trnF*, *matK*, and *rbcL*) (Fig. 1) is mostly congruent with that of Wikström et al. (2015), and all three major subfamilies (Cinchonoideae, Ixoroideae, Rubioideae) are resolved with high support. The group of primary interest, Spermacoceae (PP = 1.00), is also well supported and is sister to Knoxieae (PP = 1.00). Within Spermacoceae, the woody clades *Hedyotis* (PP = 1.00), *Kadua* (PP = 1.00) and *Arcytophyllum* (PP = 1.00) are resolved with high support as in the previous studies (Wikström et al., 2013; Neupane et al., 2015). The divergence time estimates of the major nodes are compared with Wikström et al. (2015) and Janssens et al. (2016), and summarized in Table 3. Although the age estimates of the major crown nodes are comparable to those of Wikström et al. (2015), there are some differences with respect to the Coffeeae (25.72) and Vanguerieae (26.6) alliances. The crown age of the woody clades in Spermacoceae, *Arcytophyllum*, *Hedyotis*, and *Kadua* are estimated to be 14 Ma, 12 Ma, and 7 Ma, respectively.

Spermacoceae phylogeny (Fig. 2A; online Appendix S5) based on five genes (ITS, ETS, *petD*, *rps16*, and *trnL-trnF*) is largely congruent with previous studies (Kårehed et al., 2008; Groeninckx et al., 2009; Wikström et al., 2013; Neupane et al., 2015) in resolving major clades. The clades resolved in the study are *Edrastima-Agathisanthemum/Lelya* (PP = 1.00), *Hedyotis* (PP = 1.00), *Dentella-Pentodon* (PP = 1.00), *Kohautia* (PP = 1.00), *Pentanopsis* (PP = 1.00), *Neanotis* (PP = 1.00), *Arcytophyllum-Stenotis-Houstonia/Stenaria* (PP = 0.99), an Asian-Pacific clade containing the genera *Scleromitrion/Kadua/Leptopetalum/Dimetia/Exallage/Debia/Involucrella* (PP = 1.00) with all the clades inside it PP > 0.99, and a clade containing the genera *Cordylostigma /Oldenlandia/Spermacoce* (PP = 1.00). Although sampling was increased from 14 (Kårehed et al., 2008; Groeninckx et al., 2009) to 34 species in our analysis, the relationship within the American *Arcytophyllum-Houstonia/Stenaria-Stenotis* group was unresolved (Appendix S5). Particularly, the position of the predominantly Baja Californian *Stenotis* with respect to *Arcytophyllum* and *Houstonia*/*Stenaria*, remains unclear. For this group, our DNA matrix lacked ETS and *petD* data; therefore, future study will require including these regions, and possibly other phylogenetically informative markers to resolve the position of *Stenotis* within the *Arcytophyllum-Houstonia/Stenaria-Stenotis* group. The alignments, the BEAST configuration files, and the results from BEAST analysis are available in the Dryad Digital Repository (data available from the Dryad Digital Repository: http://dx.doi.org/10.5061/dryad.81nm9).

Ancestral state estimation, trait correlation, and trait-dependent diversification—BayesTraits ancestral state estimation of life forms using phylogenetic trees obtained from the BEAST analysis suggests the herbaceous form as the ancestral state of Spermacoceae (probability of the herbaceous form at the root node = 0.90; Fig. 2A). The estimated probabilities of the herbaceous forms at the nodes of clades involving the woody *Arcytophyllum*, *Hedyotis*, *Kadua*, and their respective sister herbaceous groups were 0.85, 0.95, and 0.98, respectively. This result provides strong support for multiple origins of woody forms from herbaceous ancestors in the three major woody Spermacoceae clades (Fig. 2A). Similarly, the ancestral state estimation of habitat type in clades involving the woody groups suggests that two of the groups (*Hedyotis* and *Kadua*) had a lowland or widespread ancestral distribution (probabilities 0.90 and 0.96, respectively, Fig. 2A), whereas for *Arcytophyllum* and the related groups, the ancestral lineages may already have acquired the upland/range-restricted trait before forming the woody clade (probability 0.84, Fig. 2A). Furthermore, when habitat forms were divided into a binary state with the presence or absence of tropical upland forms, the log-marginal likelihood comparisons between the discrete independent (−158.03) and the dependent

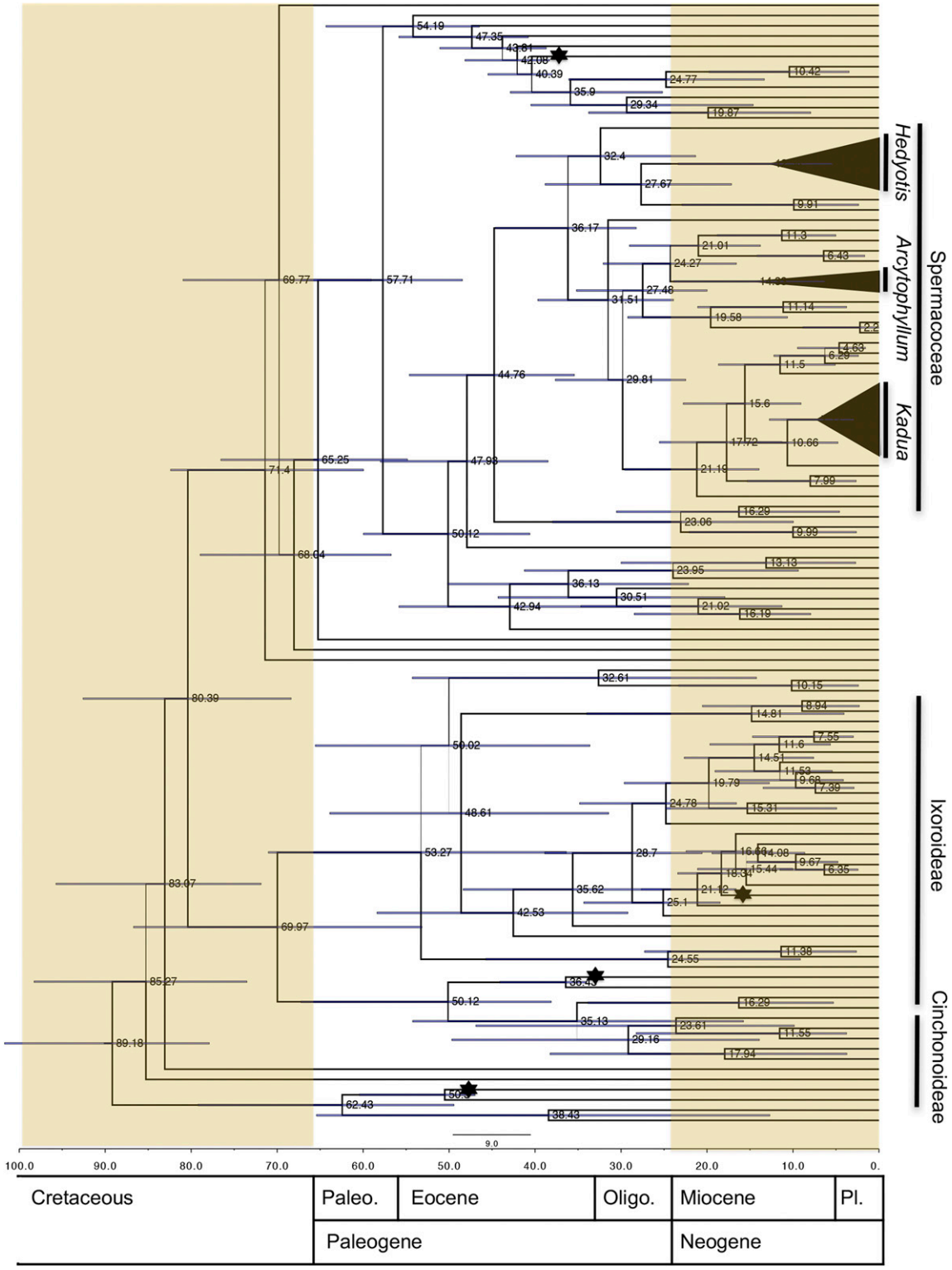

FIGURE 1 Chronogram estimated from the BEAST analysis resulting from the concatenated matrix of four plastid gene sequences (*matK*, *rbcL*, *rps16*, *trnL-trnF*) comprising 103 taxa from Rubiaceae family. The tree represents a maximum-clade credibility tree, the values and the bars at the nodes represent the median divergence times in million years ago and credibility intervals estimated by the 95% highest posterior density. The thickness of the branches on the tree represent the node support where thin lines have posterior probability <90%. The star symbol represents exponentially distributed fossil calibration placed on the members each representing from three tribes (Rubioideae: *Morinda chinensis*, offset 38 Ma; Ixoroideae: *Scyphiphora*, offset 16 Ma; and Cinchonoideae: *Cephalanthus pusillus,* offset 34 Ma), and the outgroup member (Apocynaceae: *Alstonia*, offset 47 Ma). Additionally, a normally distributed secondary root calibration (Gentianales crown age) with mean 75 Ma and standard deviation of 7.7 was enforced as done in Wikström et al. (2015).

TABLE 3. Age estimation for the major clades obtained in the present BEAST analysis and by Wikström et al. (2015) and Janssens et al. (2016).

	Present study	Wikström et al. (2015)	Janssens et al. (2016)
Clade/Group	Mean crown age, Myr (95% HPD)	Mean crown age, Myr (95% HPD)	Mean crown age, Myr (95% HPD)
Gentianales	89 (78–102)	96 (86–104)	—
Rubiaceae	85 (74–98)	87 (78–96)	~76
Cinchonoideae	50 (38–67)	51 (40–62)	—
Ixoroideae	53 (36–71)	59 (47–72)	—
Rubioideae	71 (60–82)	75 (66–84)	—
Spermacoce alliance	50 (41–60)	47 (39–57)	—
Spermacoceae	36 (28–45)	—	~36
Arcytophyllum	14 (6–24)	—	~12
Hedyotis	12 (3–17)	—	~10
Kadua	7 (3–13)	—	~5

(−147.44) models indicate the dependent model is favored over the independent model (Bayes factor > 10 log differences). This finding suggests that transitions between states in the two traits (habit and habitat) are correlated: changes of state in one trait are contingent upon the state of another trait. The comparisons among the eight averaged transition rates (from the sampled posterior distribution of rates, Fig. 2B) show that the transition of herbaceous-tropical upland forms to herbaceous-other forms was the highest (88 times higher than the opposite direction). Similarly, the average rate of transition to woody tropical upland forms was 18 times higher than the opposite direction. These transitions indicate that woody forms favor tropical upland habitats, whereas herbaceous forms favor other habitat forms (lowland, temperate upland, or a mix with widespread distribution covering both lowland and highland), exhibited by higher forward, as opposed to reverse, transition rates. The higher transitions to herbaceous-other forms and the woody tropical upland forms also indicate that woody forms persist (i.e., expected time until state change, see Zanne et al., 2014) longer in tropical upland habitats (Fig. 2B), whereas herbaceous forms tend to persist longer in other habitats (lowland, temperate upland, or a mix with widespread distribution covering both lowland and highland; Fig. 2B).

For the trait-dependent diversification analyses on habit and habitat, the best models based on Akaike weights suggest character-dependent diversification for both traits, but without any direct effects of the observed traits on their diversification heterogeneity (i.e., HiSSE models performed better than the BiSSE or the CID models). In the best models for both traits, the extinction fractions (μ/λ) were equal, and dual transitions (e.g., q0A ↔ q1B) were disallowed. For habit data (herbaceous = 0, woody = 1), in the best HiSSE model (Model 10, Table 4), the turnover rates ($\lambda + \mu$) were free, but transition rates among states were equal among all four states (i.e., observed states and their associated hidden states combination; 0A, 1A, 0B, 1B). This model performed better than any of the BiSSE models (Model 1–Model 4; Akaike weight < 0.001) suggesting that the higher diversification rate observed for woody lineages (Fig. 3A) is probably due to the hidden state (A) associated with some of the woody lineages that had exceptionally high diversification rates (rate 1A = 0.45, Fig. 3B) compared with the woody state that is not associated with the high rate hidden state (rate 1B = 0.041, Fig. 3B). The herbaceous lineages associated with the high rate hidden state also exhibited an elevated diversification rate (rate 0A = 0.32, Fig. 3B). For the habitat data (other = 0, tropical upland = 1) analysis, in the best HiSSE model (Model 24, Table 5), only turnover rates of the states associated with one of the hidden states (0B and 1B) were free, and transitions between 0B and 1B were disallowed. As in habit data, the HiSSE model performed better than any of the BiSSE models, suggesting that the higher diversification rate observed for upland lineages is due to the hidden state B associated with some of the upland lineages (Fig. 3C, D). The rates estimated for both tropical upland and other lineages associated with the hidden state A were indistinguishable (rate 0A = rate 1A = 0.34, Fig. 3D). The hidden state B had an accelerated effect on diversification rate for some tropical upland lineages (rate 1B = 0.84, Fig. 3D), whereas for other lineages, the hidden state had a negative effect on diversification rates (rate 0B = 0.12, Fig. 3D). The results from BayesTraits and HiSEE analyses are available in the Dryad Digital Repository (data available from the Dryad Digital Repository: http://dx.doi.org/10.5061/dryad.81nm9).

Wood anatomical results—Details of the wood anatomical observations are provided in Appendix S4, and examples of variations in the vessel distribution and thickness of tracheids are in Fig. 4A–D. Rays were uniserate (some biserate in *H. dendroides*) and consisted of upright or square cells (Fig. 4E–H). However, in *H. articularis* subsp. *santapaui* the ray cells were composed of mostly upright, few square and very few procumbent cells. The length-on-age curves for vessel elements were either flat (Fig. 5A, E, F) or decreasing (Fig. 5B–D) for all seven specimens measured. Although all members of *Arcytophyllum*, *Hedyotis*, *Kadua* are woody, there are huge differences in life form between the tallest (*Nesohedyotis arborea* reaching 7 m) and the smallest species that are often reported as small annual (*Hedyotis kurzii*) or perennial (*Hedyotis tetrangularis*) herbs. However, cross sections of the two latter species (Fig. 6A, C) still show considerable wood formation in the stems, outcompeting wood formation in the perennial herbs belonging to the suffrutescent clade such as *Agathisanthemum bojeri* Koltzch and *Exallage auricularia* (Fig. 6B, D). Therefore, these *Hedyotis* species are treated as woody in our analyses, although it is clear that wood formation in these particular species has been significantly reduced and may show a tendency toward secondary herbaceousness.

Historical biogeographic analysis—BioGeoBEARS model comparison identified DEC+J and BAYAREA+J as the best fitting models for biogeographic analysis of *Hedyotis* and its sister groups and of *Kadua* and its sister groups, respectively. Under the DEC+J model, *Hedyotis* and its sister clades originated in Africa/Madagascar ($P = 0.98$, Fig. 7), while the inferred ancestral range for Asian-Pacific *Hedyotis* was in Southeast Asia, west of Wallace's line (Southeast Asia, Sundaland, and Southeast China, the inferred the ancestral range for this region with $P = 0.80$, Fig. 7). From Southeast Asia, multiple colonization events occurred to other areas (Fig. 7). The Indian and Sri Lankan *Hedyotis* species were the result of separate colonization events out of Southeast Asia (west of Wallace's line). Similarly, there have been at least two colonization events out of Southeast Asia across Wallace's line to Melanesia. The ancestral range for *Kadua-Leptopetalum* was inferred to be Asia ($P = 0.90$) under the BAYAREA+J model with the inferred origin of *Kadua* in Hawaii and its subsequent dispersal to French-Polynesia (Fig. 8). Unlike in the Janssens et al. (2016) study, there was a lack of support for the clade including the American *Arcytophyllum-Houstonia/Stenaria* group and the predominately African-tropical

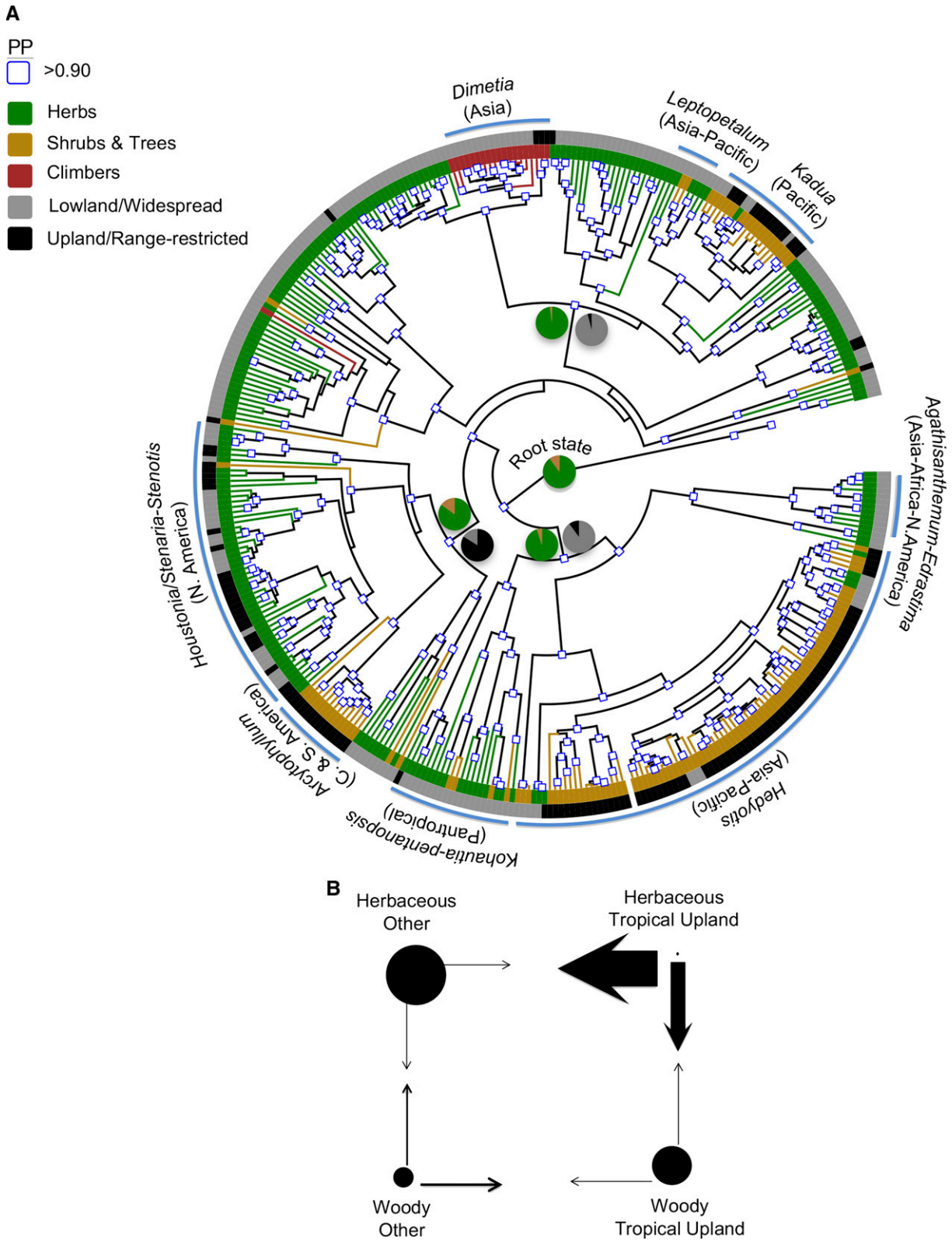

FIGURE 2 Evolution of woodiness and diversification pattern of *Arcytophyllum*, *Hedyotis*, and *Kadua* in the tribe Spermacoceae. (A) Phylogeny of the tribe Spermacoceae estimated from the BEAST analysis resulting from the concatenated matrix of two nuclear (ITS and ETS) and three plastid (*petD*, *rps16*, and *trnL-trnF*) genes sequences. The nodes with blue squares represent Bayesian posterior probabilities (PP) >0.90. The colors of the leaf and the tip (inner circle) indicate the habit type (green = herbaceous, golden = woody, and red = woody climbers) of the sampled taxon; the colors on the outer circle represent the habitat/distribution type (lowland/widespread = gray, upland/range-restricted = black) of the corresponding sampled

TABLE 4. Comparison of alternative models of trait dependent and trait independent diversification scenarios for herbaceous (0) vs. woody (1) clades in Spermacoceae, with the best model based on ΔAIC and Akaike weights (wi) italicized. These models were specified by constraining different diversification parameters (τ = turnover rate, ε = extinction fraction, q = transition rate).

Model	np	lnLik	AIC	ΔAIC	wi
BiSSE: all free	6	−938.1816	1888.363	38.233	0.00000
BiSSE: ε0=ε1	5	−938.205	1886.41	36.28	0.00000
BiSSE: q's equal	5	−938.1845	1886.369	36.239	0.00000
BiSSE: ε0=ε1, q's equal	4	−938.2394	1884.479	34.349	0.00000
CID-2: q's equal	5	−924.9289	1859.858	9.728	0.00703
CID-2: ε's, q's equal	4	−924.9561	1857.912	7.782	0.01861
CID-4: q's equal	9	−922.9966	1863.993	13.863	0.00089
CID-4: ε's equal, q's equal	6	−923.2976	1858.595	8.465	0.01323
HiSSE: q's equal	9	−919.0332	1856.066	5.936	0.04685
HiSSE: ε's equal, q's equal	*6*	*−919.065*	*1850.13*	*0*	*0.91132*
HiSSE: τ0A=τ1A=τ0B, ε0A=ε1A=ε0B, q's equal	5	−932.1253	1874.251	24.121	0.00001
HiSSE: τ0A=τ1A=τ0B, ε's equal, q's equal	4	−932.4205	1872.841	22.711	0.00001
HiSSE: q0B1B = 0, q1B0B = 0, all other q's equal	9	−926.0499	1870.1	19.97	0.00004
HiSSE: ε's equal, q0B1B = 0, q1B0B = 0, all other q's equal	6	−927.4998	1867	16.87	0.00020
HiSSE: τ0A=τ1A=τ0B, ε0A=ε1A=ε0B, q0B1B = 0, q1B0B = 0, all other q's equal	5	−935.0425	1880.085	29.955	0.00000
HiSSE: τ0A=τ1A=τ0B, ε's equal, q0B1B = 0, q1B0B = 0, all other q's equal	4	−938.5291	1885.058	34.928	0.00000
HiSSE: τ0A=τ0B, ε0A=ε0B, q's equal	7	−926.9963	1867.993	17.863	0.00012
HiSSE: τ0A=τ0B, ε's equal, q's equal	5	−927.6964	1865.393	15.263	0.00044
HiSSE: τ0A=τ0B, ε0A=ε0B, q0B1B = 0, q1B0B = 0, all other q's equal	7	−935.0661	1884.132	34.002	0.00000
HiSSE: τ0A=τ0B, ε's equal, q0B1B = 0, q1B0B = 0, all other q's equal	5	−935.3146	1880.629	30.499	0.00000
HiSSE: τ0A=τ1A, ε0A=ε1A, q's equal	7	−929.2614	1872.523	22.393	0.00001
HiSSE: τ0A=τ1A, ε's equal, q's equal	5	−926.6736	1863.347	13.217	0.00123
HiSSE: τ0A=τ1A, ε0A=ε1A, q0B1B = 0, q1B0B = 0, all other q's equal	7	−931.8134	1877.627	27.497	0.00000
HiSSE: τ0A=τ1A, ε's equal, q0B1B = 0, q1B0B = 0, all other q's equal	5	−931.9575	1873.915	23.785	0.00001

American *Spermacoce-Oldenlandia* group (Appendix S5). Furthermore, the position of our newly added members from the *Stenotis* group was unresolved within the *Arcytophyllum-Houstonia/Stenaria* group, which could affect the ancestral range estimation. Therefore, biogeographic analysis was not performed for *Arcytophyllum* and its related groups. The results from BioGeoBEARS analysis are available in the Dryad Digital Repository (data available from the Dryad Digital Repository: http://dx.doi.org/10.5061/dryad.81nm9).

DISCUSSION

***Timing and origin of the woody* Arcytophyllum, Hedyotis, *and* Kadua**—Our time-calibrated chronogram, based on the best-sampled Spermacoceae data set generated so far, convincingly shows an independent origin of the woody *Arcytophyllum*, *Hedyotis*, and *Kadua* clades (Fig. 1). This supports the general idea that secondary woodiness has arisen multiple times during evolution, even within the same group (Lens et al., 2009, 2012a). *Arcytophyllum* and *Hedyotis* originated and diversified almost simultaneously during the middle/late Oligocene and Miocene, yet on different continents (see next paragraph), while the origin and diversification events in *Kadua* started later in the Miocene (Fig. 1). The timing of the origin of these tropical upland forms and the diversification coincides with many other major radiations documented during the Miocene and Pliocene (e.g., Koenen et al., 2015; Hughes and Atchison, 2015; Breitkopf et al., 2015; Spriggs et al., 2015; see also the editorial by Hughes et al., 2015). In addition, the ancestral state analysis using BayesTraits indicates that the ancestors of the three woody clades were herbaceous, meaning that a life-form shift from herbaceous toward derived/secondary woodiness took place independently (Fig. 2A). Our expanded anatomical observations support this secondarily derived origin of woodiness and provide sound evidence for protracted juvenilism in *Arcytophyllum*, *Hedyotis*, and *Kadua* (cf. Lens et al., 2009). Examples include the presence of predominantly upright or upright-to-square ray cells (Fig. 4E–H) and decreasing or flat length vs. age curves (Fig. 5) for vessel elements.

The historical biogeographic analyses suggest that the ancestors of Asian-Pacific *Hedyotis* and Hawaiian-French Polynesian *Kadua* came from elsewhere, but the ancestral range for the Andean *Arcytophyllum* could not be ascertained due to the lack of resolution for the North American *Stenotis* clade with respect to the South American *Arcytophyllum* and the North American *Houstonia/Stenaria* group. The inferred ancestral range for *Hedyotis* and its allies (*Agathisanthemum-Edrastima*, *Pentodon-Dentella*, and *Kohautia-Pentanopsis*) in Africa/Madagascar suggests at least three colonization events out of Africa/Madagascar to Asia, one of which led to the formation of the Asian-Pacific *Hedyotis* (Fig. 7). Similarly, the inferred ancestral range estimates for the *Kadua-Leptopetalum*

taxon. Pie charts on the nodes represent the probability of woody (golden) vs. herbaceous (green) states, and the lowland/widespread (gray) vs. upland/range-restricted (black) forms obtained from the ancestral state estimation for the nodes involving the woody clades of interest (*Arcytophyllum*, *Hedyotis*, and *Kadua*) based on the BayesTraits Multistate (model) RJ MCMC analysis on the 10,000 Spermacoceae posterior trees. (B) Schematic diagram of the BayesTraits Dependent (model) RJ MCMC analysis on the 10,000 Spermacoceae posterior trees showing relative rates of transitions among traits contingent upon another trait where the arrow width are scaled to their average transition rates, and the size of each circle is scaled to the persistence time (inverse of the sum of transition rates away from a given character state) in that state.

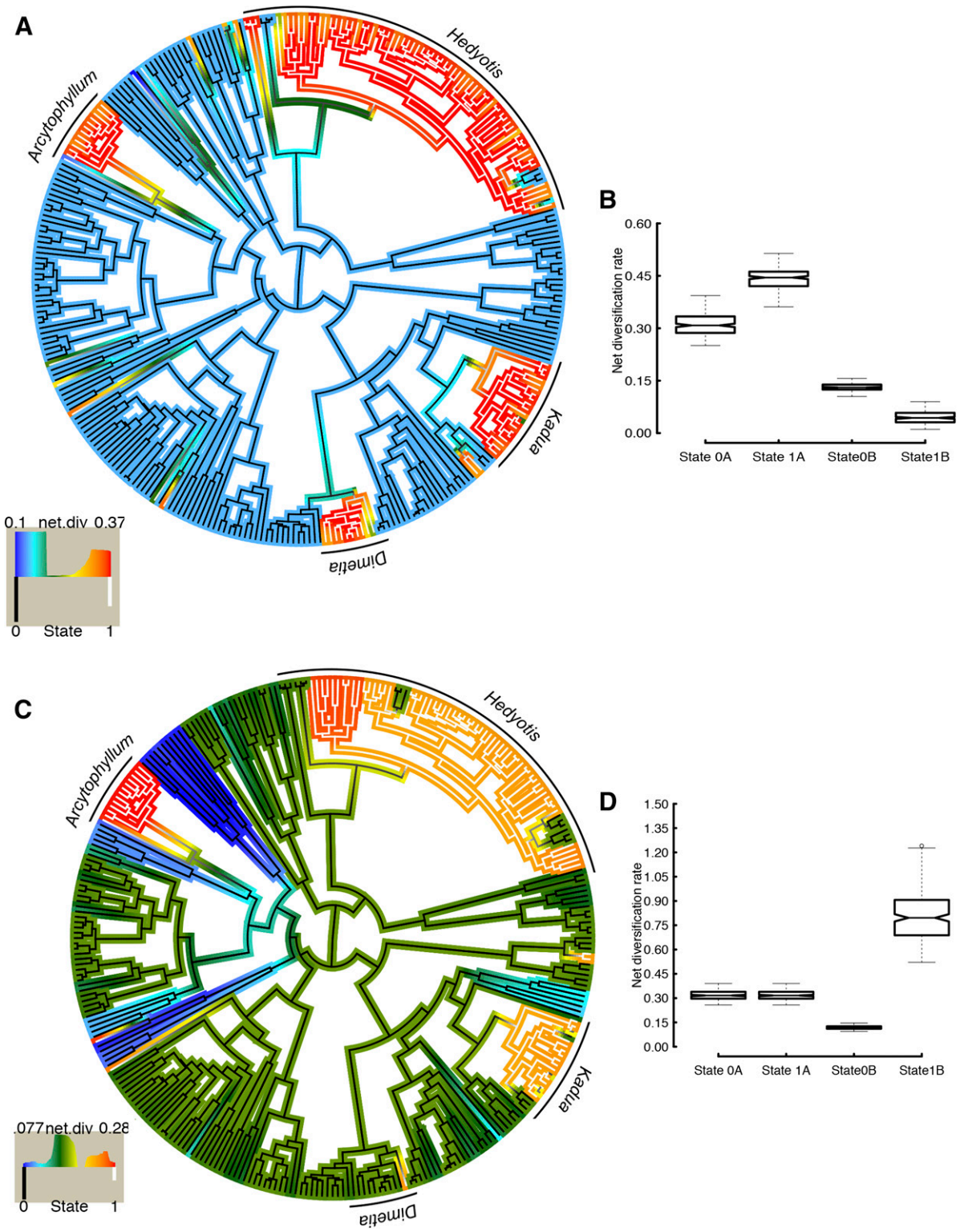

FIGURE 3 Hidden state speciation and extinction (HiSSE) results for habit and habitat data. (A) HiSSE reconstruction of character state estimation and net diversification rates of herbaceous and woody state lineages on Spermacoceae phylogeny under the best model (HiSSE Model 10) based on the model-averaged parameter estimates from the set of all 24 models. (B) Rate estimates and their estimates of confidence intervals for each of the four state combinations under HiSSE Model 10 for habit trait analysis. (C) HiSSE reconstruction of character state estimation and net diversification rates of tropical upland and other state lineages on Spermacoceae phylogeny under the best model (HiSSE Model 24) based on the model-averaged parameter estimates from the set of all 24 models. (D) Rate estimates and their estimates of confidence intervals for each of the four state combinations under HiSSE Model 24 for habitat trait analysis. The histogram shows a gradient of net diversification rates estimates (x-axis) with the frequency of sampled lineages for each rate and state category (y-axis).

TABLE 5. Comparison of alternative models of trait dependent and trait independent diversification scenarios for other (0) vs. tropical upland (1) clades in Spermacoceae, with the best model based on ΔAIC and Akaike weights (wi) italicized. These models were specified by constraining different diversification parameters (τ = turnover rate, ε = extinction fraction, q = transition rate).

Model	np	lnLik	AIC	ΔAIC	wi
BiSSE: all free	6	−927.663	1867.326	19.981	0.000043
BiSSE: ε0=ε1	5	−927.9743	1865.949	18.604	0.000085
BiSSE: q's equal	5	−933.0419	1876.084	28.739	0.000001
BiSSE: ε0=ε1, q's equal	4	−934.6949	1877.39	30.045	0
CID-2: q's equal	5	−926.0228	1862.046	14.701	0.000601
CID-2: ε's, q's equal	4	−926.0271	1860.054	12.709	0.001626
CID-4: q's equal	9	−923.3056	1864.611	17.266	0.000167
CID-4: ε's equal, q's equal	6	−924.2152	1860.43	13.085	0.001347
HiSSE: q's equal	9	−922.0698	1862.14	14.795	0.000573
HiSSE: ε's equal, q's equal	6	−926.7325	1865.465	18.12	0.000109
HiSSE: τ0A=τ1A=τ0B, ε0A=ε1A=ε0B, q's equal	5	−932.1274	1874.255	26.91	0.000001
HiSSE: τ0A=τ1A=τ0B, ε's equal, q's equal	4	−933.9559	1875.912	28.567	0.000001
HiSSE: q0B1B = 0, q1B0B = 0, all other q's equal	9	−922.6739	1863.348	16.003	0.000313
HiSSE: ε's equal, q0B1B = 0, q1B0B = 0, all other q's equal	6	−926.0339	1864.068	16.723	0.000219
HiSSE: τ0A=τ1A=τ0B, ε0A=ε1A=ε0B, q0B1B = 0, q1B0B = 0, all other q's equal	5	−935.5394	1881.079	33.734	0
HiSSE: τ0A=τ1A=τ0B, ε's equal, q0B1B = 0, q1B0B = 0, all other q's equal	4	−935.6469	1879.294	31.949	0
HiSSE: τ0A=τ0B, ε0A=ε0B, q's equal	7	−928.6848	1871.37	24.025	0.000006
HiSSE: τ0A=τ0B, ε's equal, q's equal	5	−933.7365	1877.473	30.128	0
HiSSE: τ0A=τ0B, ε0A=ε0B, q0B1B = 0, q1B0B = 0, all other q's equal	7	−925.6889	1865.378	18.033	0.000114
HiSSE: τ0A=τ0B, ε's equal, q0B1B = 0, q1B0B = 0, all other q's equal	5	−927.3615	1864.723	17.378	0.000157
HiSSE: τ0A=τ1A, ε0A=ε1A, q's equal	7	−920.0182	1854.036	6.691	0.032952
HiSSE: τ0A=τ1A, ε's equal, q's equal	5	−925.0548	1860.11	12.765	0.001581
HiSSE: τ0A=τ1A, ε0A=ε1A, q0B1B = 0, q1B0B = 0, all other q's equal	7	−920.2905	1854.581	7.236	0.025092
HiSSE: τ0A=τ1A, ε's equal, q0B1B = 0, q1B0B = 0, all other q's equal	*5*	*-918.6723*	*1847.345*	*0*	*0.935013*

group as continental Asia and for *Kadua* as the Hawaiian Islands suggests a colonization event into the Hawaiian Islands leading to its radiation on the islands (Fig. 8). The colonization of the ancestral form of the Andean *Arcytophyllum* and its subsequent diversification could have happened anywhere in the lower elevation zone of the Northern Andean or in the northern part of the Central Andean region covering the current Páramos region (its current distribution; see details in the next section about dispersal dynamics).

***Historical dispersal dynamics of the three woody clades: Into and inside of Asia-Pacific* (Hedyotis), *into and out of Hawaii* (Kadua), *and into the Andes* (Arcytophyllum)**—The split age of *Agathisanthemum-Edrastima* and *Hedyotis* at ~27 Ma with a long lag time until the crown age of *Hedyotis* (~13 Ma) from the Rubiaceae chronogram (Fig. 1), or ~19 Ma from the secondarily calibrated Spermacoceae chronogram with Thai *Hedyotis corymbiformis* samples added (Appendix S5) excludes both Eocene boreotropical expansion and vicariance through the Indian plate rafting as the probable cause for the origin of the Africa/Madagascan–Asia–Pacific disjunction. Although long-distance dispersal becomes a null model for such patterns, a few studies (Cruaud et al., 2010; van Welzen et al., 2014; Zhou et al., 2012; and see Thomas et al., 2015 for detailed discussion) have highlighted an alternative path across Arabia during the Miocene climatic optimum for dispersal between Africa and continental Asia. The subsequent diversification that occurred during the Miocene (warmer period) and early Pliocene (cooler period) may have given rise to many *Hedyotis* endemics in continental Southeast Asia (~50 species), Borneo (~15 species), Southeast China (~32 species), Philippines (~32 species), Indian Western Ghats Mountains (20 species), and Sri Lanka (25 species). At least two dispersal events have occurred across Wallace's line to the New Guinean highlands (Fig. 7) during the Pliocene to Pleistocene, which is also a pattern seen among other groups of organisms (e.g., *Aglaia*, Meliaceae [Muellner et al., 2008, 2009]; *Pseuduvaria*, Annonaceae [Su and Saunders, 2009]; *Begonia* [Thomas et al., 2011]; and a meta-analysis of 61 phylogenies of plants and animals [de Bruyn et al., 2014]). The colonization of *Hedyotis* in New Guinea was likely facilitated by the formation of an optimal montane climate in the Central New Guinean highlands (timing of central orogeny of New Guinea 10–5 Ma, Hall, 2009). Two successive and independent colonizations of the southern part of the Indian Western Ghats Mountains and the Sri Lankan highlands (Fig. 7) formed remarkably narrow endemic groups (only 2 of the total 48 species are shared by two regions despite their separation by less than 100 km) that suggest important dispersal dynamics within both regions. After the first pulse of in situ diversification in the Western Ghats Mountains, the second/later immigrants could not have established in the Western Ghats probably due to niche pre-emption (see Silvertown, 2004, Silvertown et al., 2005) but were able to diversify in the Central Sri Lankan highlands due to the available open niches. Three sampled species (of 13 total) of Micronesian *Hedyotis* resolved sister to two New Guinean taxa, which in turn resolved sister to the Southeast Asian clade, suggesting at least one dispersal out of New Guinea to the Pacific (Micronesian Islands). However, no definitive conclusions can be made regarding the number and directionality of their recruitment due to the lack of enough samples from that region.

The crown age of *Kadua* (median age ~7 Ma, 95% HPD 3–13 Ma, Fig. 1) implies that the colonization from continental Asia may have happened right before or after the emergence of the current oldest island (*Kauai*, 5.1 Ma). A similar Asian origin for some Hawaiian taxa has been inferred in other studies (e.g., Givnish et al., 2009; Cronk et al., 2005; Clark et al., 2009). Kauai contains deeply diverging and endemic lineages (see Parker, 2012) such as *Kadua tryblium* (D.R.Herbst & W.L.Wagner) W.L.Wagner & Lorence (section *Phyllozygia* sensu Terrell et al., 2005), and *Kadua st-johnii*

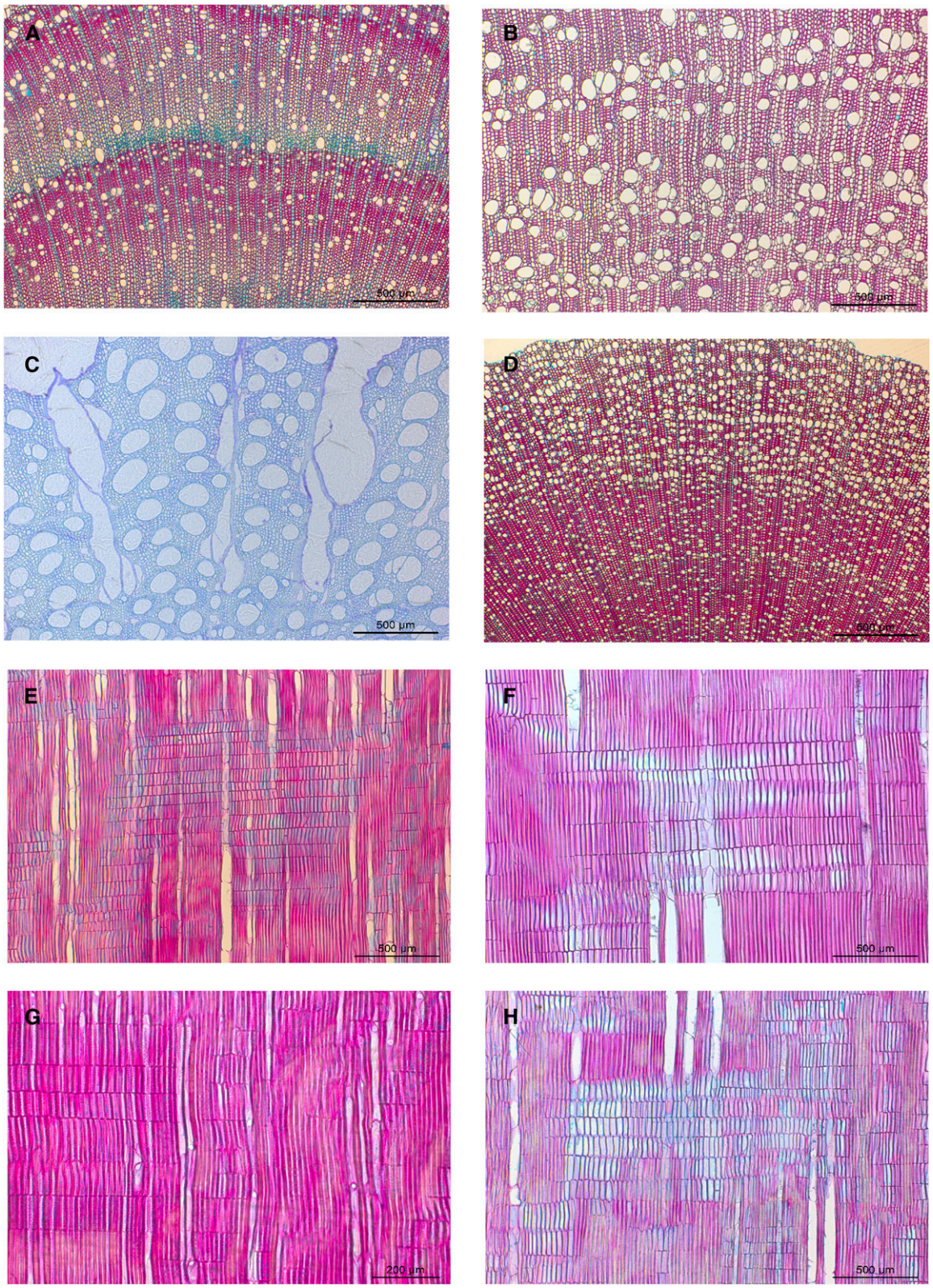

FIGURE 4 Light micrographs of transverse (A–D) and radial (E–H) sections showing variations in the vessel distribution, thickness of tracheids and the upright ray cells. Sections were stained with a mixture of safranin and alcian blue (35:65). (A, E) *Hedyotis corymbiformis*. (B, F) *Hedyotis dendroides*. (C) *Dimetia* sp., a climber species showing a dissected wood cylinder. (D, G) *Kadua cordata*. (H) *Hedyotis gartmorensis*.

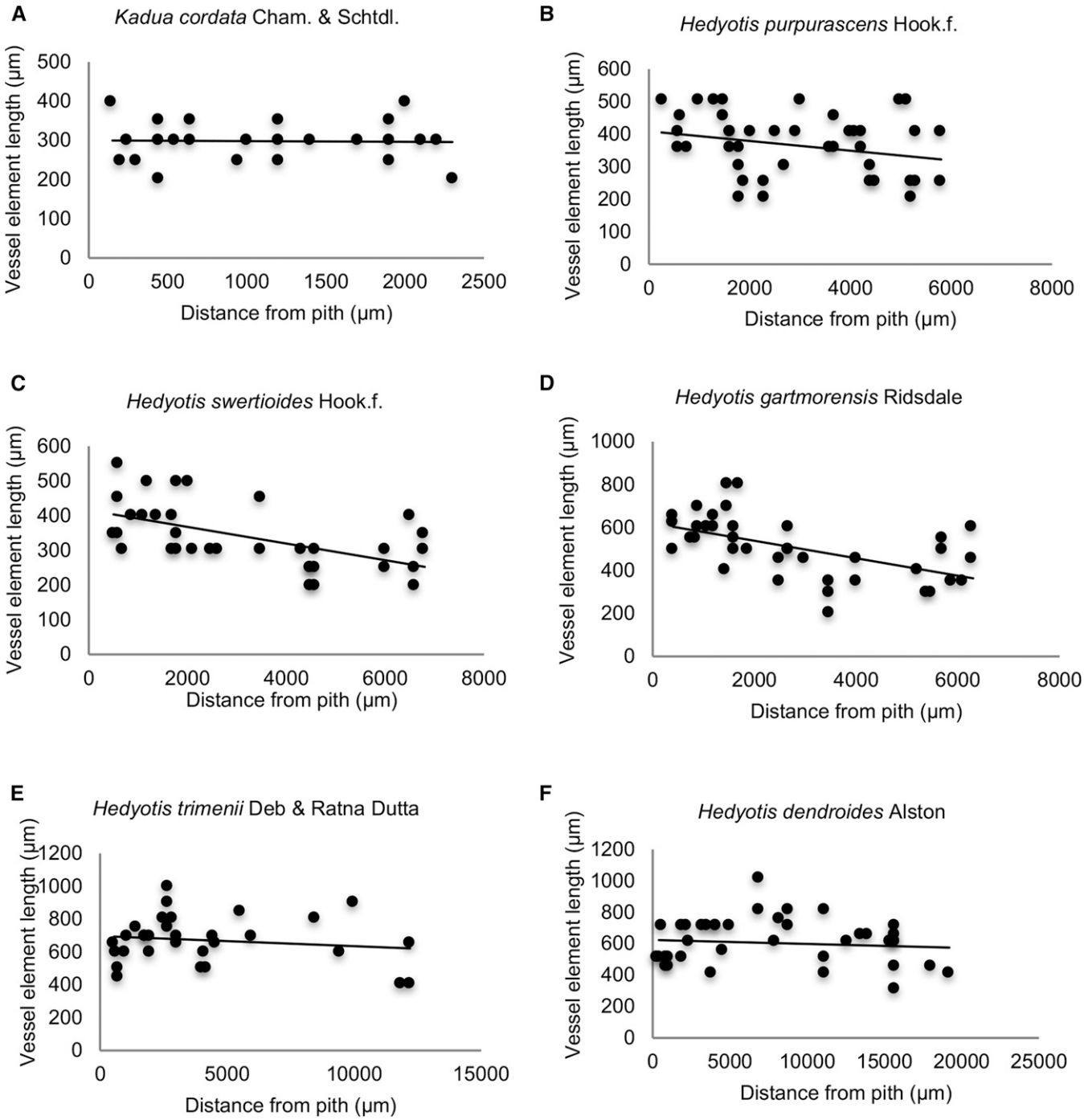

FIGURE 5 Vessel element length vs. age curves for *Kadua* (A) and *Hedyotis* (B–F) showing either flat (A, E, F) or decreasing (B–D) trend from pith toward outer cambium.

(B.C.Stone & Lane) W.L.Wagner & Lorence (in the section *Gouldiopsis* sensu Terrell et al., 2005). The second oldest island, Oahu (3.7 Ma), contains the deeply diverging endemic *K. parvula* A.Gray representing the section *Wiegmannia* (sensu Terrell et al., 2005). Similarly, Kauai supports the highest number of *Kadua* species (13 species, 6 endemics), whereas the youngest island in Hawaii contains only six *Kadua* species, none of which are endemic to the island. This pattern somewhat follows the "progression rule" (Wagner and Funk, 1995), where the colonization and the subsequent in situ diversification of the lineage follows the progressive age of the islands. The five other *Kadua* species are French Polynesian endemics that are mainly concentrated in the Marquesas (4 species) and Society (3 species) Islands. The fleshy-fruited French Polynesian *Kadua romanzoffiensis* Cham. & Schltdl. is distributed widely across the South Pacific islands (Society, Austral, Tuamotu-Gambier, Pitcairn, Cook, Samoa), unlike other members from this group, which have rather restrictive distribution in Marquesas, Society, or Rapa Iti Islands. At least one colonization from Hawaii to French

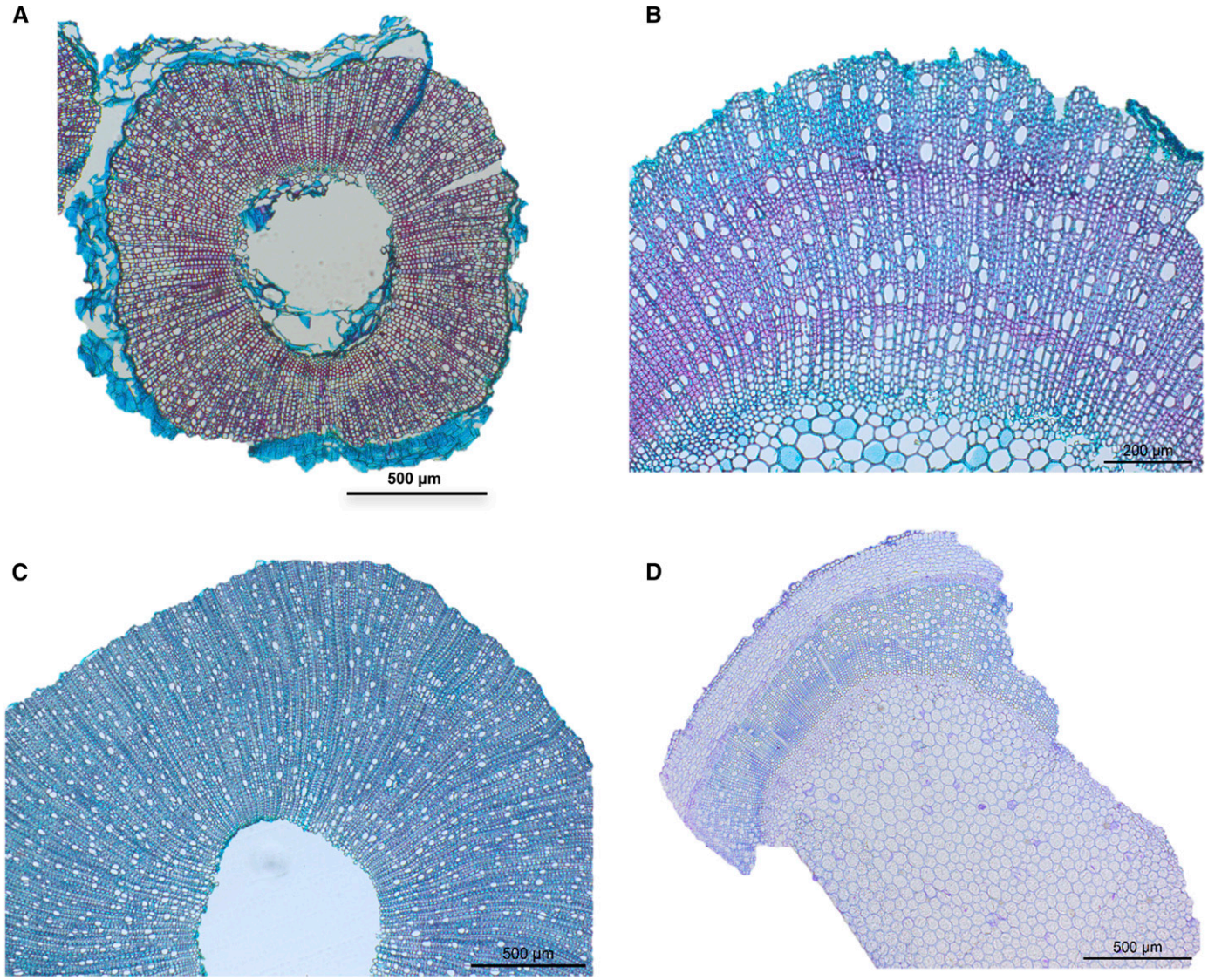

FIGURE 6 Light micrographs of transverse sections to compare secondary growth in perennial but nonwoody *Hedyotis kurzii* (A) and *H. tetrangularis* (C) with other perennial herbs in the tribe Spermacoceae *Agathisanthemum bojeri* (B), *and Exallage auricularia* (D). Sections were stained with a mixture of safranin and alcian blue (35:65).

Polynesia, leading to the section *Protokadua* (8 species), can be inferred (Fig. 8). Despite the distance of ~3860 km (Wagner, 1991), the Marquesas Islands are the closest island group to Hawaii, and the link between the two island groups, probably via bird migratory routes, has been documented in a number of studies (e.g., *Santalum* [Harbaugh and Baldwin, 2007; Harbaugh, 2008], *Bidens* [Knope et al., 2012], *Melicope* [Harbaugh et al., 2009], *Astelia* [Birch and Keeley, 2013; Appelhans et al., 2013], and *Coprosma* [Cantley et al., 2014]).

The ancestral range for the Andean *Arcytophyllum* could not be confirmed in our study. However, the arrival of the ancestral *Arcytophyllum* (between stem and crown node 24–14 Ma) to the Andes before it gained the current elevations can be indirectly inferred from the lack of any apparent phylogenetic structure showing North–South Andean disjunction across the low Amotape–Huancabamba region (southern Ecuador and northern Peru). The current widespread distribution of some *Arcytophyllum* species, unlike in many other high-elevation lineages where the Amotape–Huancabamba region was an important biogeographic barrier (e.g., *Cinchona* [Antonelli et al., 2009]; *Lithospermum* [Weigend et al., 2010]; *Macrocarpaea* [Struwe et al., 2009]; *Peperomia* [Symmank et al., 2011]), also indicates such a pattern. Furthermore, the lag time of ~7 Ma between *Arcytophyllum thymifolium* and the divergence of the rest of the sampled *Arcytophyllum* species starting ~7 Ma (Appendix S5) is also indicative of the role of the Andean orogeny in the diversification of this genus, as suggested by Luebert and Weigend (2014), for the older-than-uplift lineages where the later diversification paralleled the Andean uplift (e.g., Antonelli et al., 2009; Antonelli and Sanmartín, 2011 a, b; Struwe et al., 2009).

Shifts toward the woody life in Spermacoceae are mainly triggered in high-altitude and wet tropical ecosystems—Several hypotheses have been proposed to explain the dramatic increase in wood formation in otherwise herbaceous plant lineages, with most of these studies focusing on insular woodiness (cf. *Kadua*). According to

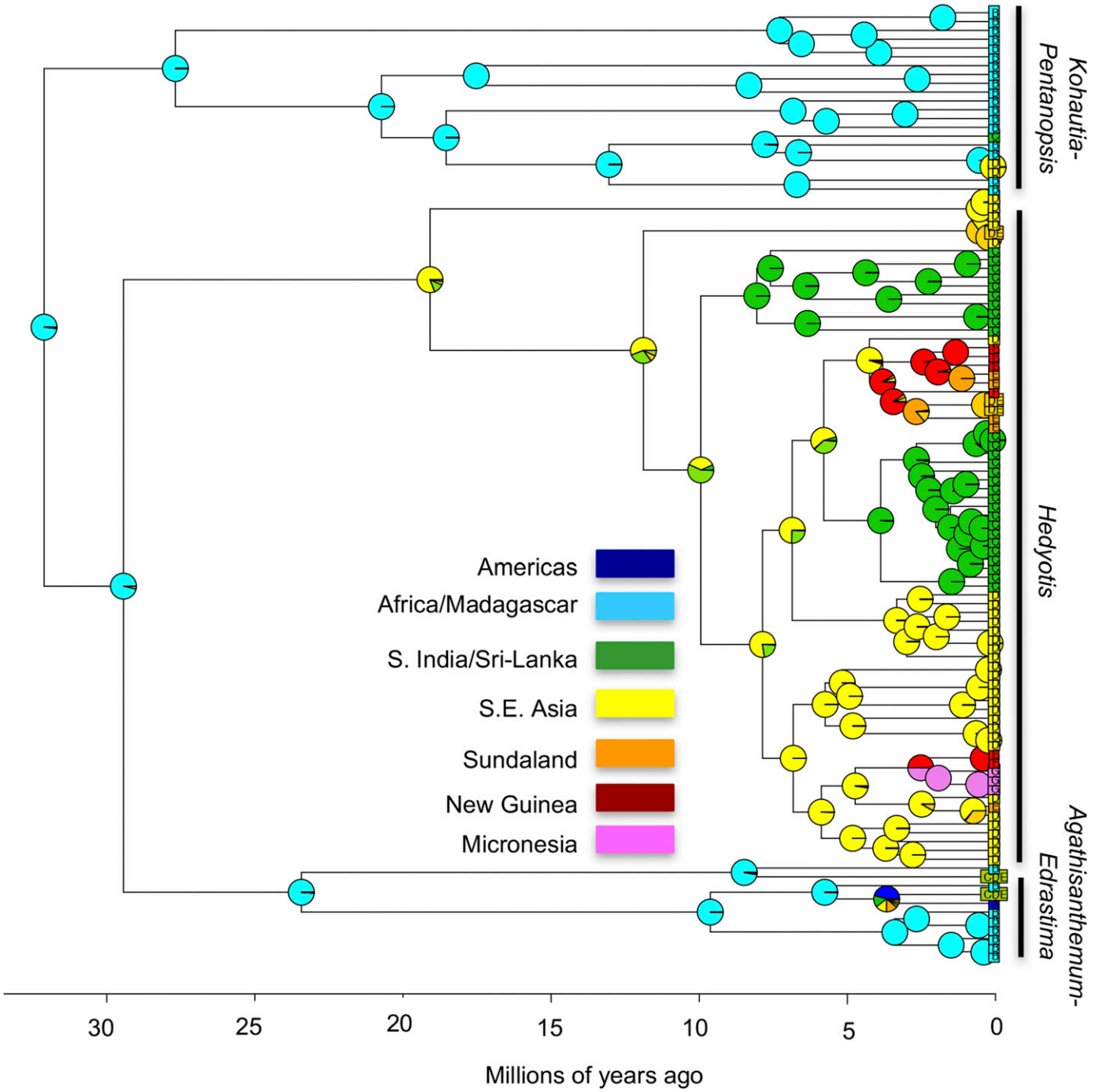

FIGURE 7 BioGeoBEARS graphical output showing ancestral range estimation on a time-calibrated *Kohautia-Hedyotis-Agathisanthemum-Edrastima* phylogeny using nuclear (ITS, ETS) and plastid (*petD*, *rps16*, *trnL-trnF*) DNA under the DEC+J model. The pie chart represents the most likely ancestral area recovered at each node by BioGeoBEARS. S. = southern; S.E. = South East.

these hypotheses, increased woodiness—often coevolving with an increased plant size and life span—could be a strategy for evolutionary success due to (1) competitive advantage for resources such as light (Darwin, 1859; Tilman, 1988; Givnish, 1995), (2) longer reproductive periods (Wallace, 1878), (3) longer growing season due to absence of large native herbivores on islands or due to the moderate climatic condition in insular settings allowing plants to grow without frost temperatures (Carlquist, 1974, 2012), or (4) better adaptation to cope with drought-induced embolism formation in the water-conducting xylem (Lens et al., 2013b). Increased woodiness in some secondarily woody Spermacoceae, i.e., *Amphistemon humbertii* Groeninckx and *Thamnoldenlandia ambovombensis* Groeninckx native to the dry parts of southwestern Madagascar, could indeed be an adaptation to drought stress, which may also be the case for many other secondarily woody angiosperms growing in deserts such as the genera *Vella* or *Zilla* (Brassicaceae), and several

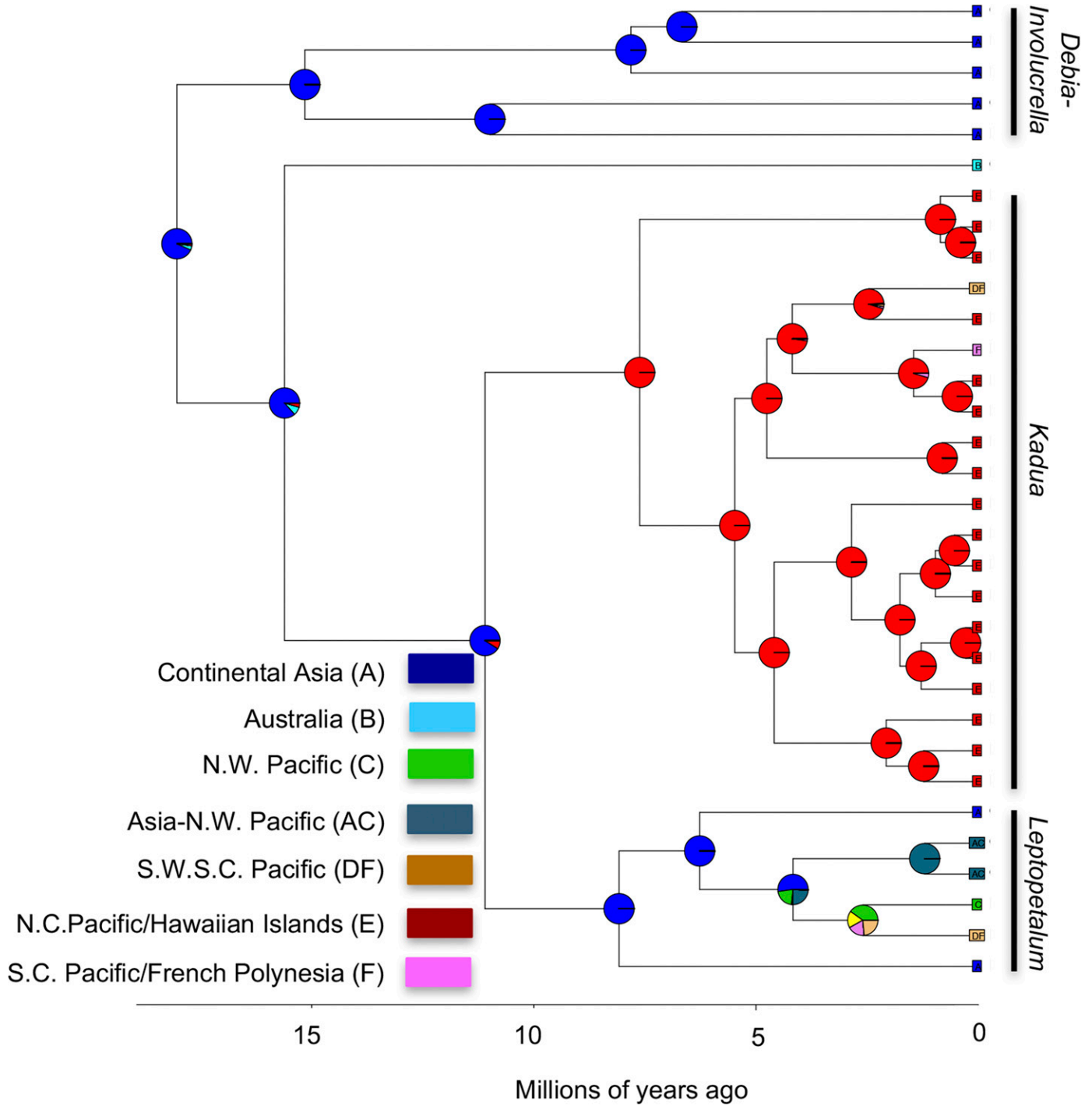

FIGURE 8 BioGeoBEARS graphical output showing ancestral range estimation on a time-calibrated *Debia/Involucrella-Kadua-Leptopetalum* phylogeny using nuclear (ITS, ETS) and plastid (*petD*, *rps16*, *trnL-trnF*) DNA under the BAYAREA+J model. The pie chart represents the most likely ancestral area recovered at each node by BioGeoBEARS. N.C. = north-central; N.W. = northwestern; S.C. = south-central; S.W. = southwestern.

of the insular woody Canary Island endemics (Lens et al., 2013a), including the daisies belonging to the genus *Argyranthemum* (Asteraceae; Lens et al., 2016). However, drought stress was probably not a limiting factor throughout evolution of the genera *Arcytophyllum*, *Hedyotis*, and *Kadua*. Instead, members of these groups are often restricted to high altitudes in the mist-covered areas of tropical montane cloud forests and upper grasslands below the frost line (Table 1). The South American *Arcytophyllum* is distributed between 1675–4250 m a.s.l. in Costa Rica and Panama, in wet (sub)paramos of the northern Andes, with a few extending to the punas [*Arcytophyllum filiforme* (Ruiz & Pav.) Standl., *A. setosum* (Ruiz & Pav.) Standl.], and the widespread *A. thymifolium* (Ruiz & Pav.) Standl. found as low as 400 m a.s.l. in the Loma Formations in coastal Peru to 4000–4500 m a.s.l. near Mojanda Lake, northern

Ecuador (Mena, 1990). *Hedyotis* species are found in the tropical highlands of Asia and the Pacific, namely, the Western Ghats (including the central highlands of Sri Lanka) Mountains, Southeast Asia (extending up to the vegetation border at 3500 m a.s.l. on Mt. Kinabalu; Merckx et al., 2015), Southeast China, Philippines, and Melanesia (New Guinean highlands). However, a few of the Pacific *Hedyotis* (the Micronesian members) can be found in the low-lying wet areas (as low as 50 m a.s.l.). Similarly, the Polynesian *Kadua* have their highest diversity in the highlands of the Hawaiian Islands (often between 500–2438 m a.s.l.), and some occur in French Polynesia (8 species, Marquesas Islands and Society Islands, 200–1067 m a.s.l.). Outside *Arcytophyllum*, *Hedyotis*, and *Kadua*, the arborescent *Nesohedyotis arborea* (Roxb.) Bremek. (Saint Helena Island, 700 m) and *Pseudonesohedyotis bremekampii* Tennant (Uluguru mountains, Tanzania >1000 m, Tennant, 1965) also grow in the damp and misty forests of the highlands.

The repeated occurrences of woody habit in the tropical upland habitat (Fig. 2A) and the results from the BayesTraits correlation analysis on habit and habitat forms (Fig. 2B) indicate that the tropical mountains may have favored evolution of the woody habit. However, the correlation study of categorical traits should be interpreted with caution due to phylogenetic pseudoreplication (Maddison and FitzJohn, 2014). Maddison and FitzJohn (2014) illustrated four possible scenarios by which a pair of traits with their binary states could evolve in a group and suggested that only two (replicated codistribution, replicated bursts) provide good evidence for their significant association. In these two scenarios, the codistribution of states of a pair of traits appears multiple times independently across the phylogeny. In our study, there are at least six such independent cases (*Arcytophyllum*, *Dimetia scandens*, *Hedyotis*, *Kadua*, *Nesohedyotis*, *Pseudonesohedyotis*) where the secondarily derived woody groups are found in the moist tropical mountains, resembling the replicated bursts model of Maddison and FitzJohn (2014).

***Diversification dynamics of the woody* Arcytophyllum, Hedyotis, *and* Kadua**—The HiSSE results suggest that the diversification within Spermacoceae lineages is not under the direct influence of either habit or habitat traits, but under some association with the state of an unobserved trait. The overall high diversification rate observed for some woody lineages (Fig. 3A, B) is due to a hidden state associated with some of the woody lineages. Similarly, the higher diversification rate observed for tropical upland lineages (Fig. 3C, D) is due to the association of some of these tropical upland lineages with a state of an unobserved trait. The switch to a perennial life form and its association with mountain habitat and diversifications have been studied previously (e.g., Arabideae [Karl and Koch, 2013]; *Lupinus* [Drummond et al., 2012b; Hughes and Atchison, 2015]). Interestingly, there are noticeable differences among the three major woody Spermacoceae lineages in terms of species diversity and species turnover despite their similarities to the pattern of habit and habitat switch. First is the difference in the species number disparity between the woody clades (*Hedyotis* ~180 species, *Kadua* 29 species, and *Arcytophyllum* 16 species) with respect to their sister lineages (*Agathisanthemum-Edrastima* 10 species, *Leptopetalum* 8 species, *Houstonia/Stenaria-Stenotis* 36 species, respectively). In other words, the extant species number is disproportionately higher in *Hedyotis* and *Kadua* compared with their closest relatives, while the situation for *Arcytophyllum* vs *Houstonia/Stenaria-Stenotis* is completely different without any apparent reason. Second, unlike *Kadua* where the members of the related *Leptopetalum* are distributed in the lowlands (<500 m a.s.l.), members in the sister lineages of *Arcytophyllum* and *Hedyotis* can also be found at high altitudes [e.g., *Houstonia serpyllifolia* Michx., *H. purpurea* var. *montana* (Small) Terrell at 1000–2000 m a.s.l. in the southern Appalachian Mountains, *H.* section *Ericotis* and *Stenaria* at 690–4000 m a.s.l., *Stenotis* at 2450 m a.s.l. in the arid highlands of the southwestern United States and northern Mexico, and *Agathisanthemum* and *Lelya* at 2000 m a.s.l. in tropical African highlands; Terrell, 1996, 2001a, 2001b; Verdcourt, 1976, 1989]. Third, there is at least one deeply nested lineage in the *Arcytophyllum* and *Hedyotis* groups (*Arcytophyllum thymifolium* and Thai *Hedyotis corymbiformis* Geddes group, online Appendix S5) and a pause (~7 Ma and ~4 Ma, respectively) before these groups experienced a rapid diversification. Therefore, unlike the *Lupinus* diversification reported by Drummond et al. (2012b), simply acquiring perenniality with upland distribution might not be a guaranteed pathway to triggering radiation in any given group. The diversification dynamics in Asia and the Pacific (Micronesia) for *Hedyotis*, Polynesia for *Kadua*, and Andes for *Arcytophyllum* appear to be highly context dependent and include timing and interplay of a range of intrinsic and ecological variables (See Donoghue and Sanderson, 2015).

For instance, unlike *Hedyotis* and *Kadua*, where the ancestral forms were most likely lowland/widespread taxa, the ancestors of *Arcytophyllum* may already have been adapted to the upland habitat (Fig. 2A) where the preadapted montane ancestral forms (from outside tropics) later colonized the rising Andes and subsequently gained the woody habit along the moist tropical montane system where such extended frost is absent but has dramatic fluctuations in diurnal temperature ("summer every day and winter every night", Hedberg, 1964). Therefore, acquiring higher altitudes other than wet tropical mountains may not necessarily induce perenniality of a large stature seen in the three woody groups, and acquisition of perennial form may not always result in rapid radiation and a high net species number in the tropical mountains. So, what explains the high species diversity/richness in *Hedyotis* and *Kadua* and lack thereof in *Arcytophyllum*, despite the Andes being the region shown to have the highest rate of diversification (Madriñán et al., 2013)? The difference in species diversity is hard to determine, but we assume that in addition to the altitudinal allopatric process, the Asia-Pacific region—with its large size and area heterogeneity combined with the different ages of land masses due to its complex geological history (Fine and Ree, 2006; Slik et al., 2015)—produces a combination of temporal, spatial, and altitudinal isolating mechanisms on the noncontiguous areas that may have helped *Hedyotis* and *Kadua* to accumulate more species than in the Andes. The temporal factor (the oldest Western Ghats mountains to the youngest Kinabalu mountains) could have allowed the preadapted species to colonize new areas with open niches/habitat at different times and speciate there (see Donoghue and Sanderson, 2015; Merckx et al., 2015). In other words, the current *Arcytophyllum* diversity may be the result of one single pulse of diversification along the Andes orogeny of the mountain (vertical speciation as shown by Merckx et al., 2015), followed by dispersal to create widespread distributions. *Hedyotis* and *Kadua,* on the other hand, experienced several such pulses of radiations due to noncontiguous landmasses separated by temporal and physical barriers (different age of emerging islands and colliding landmasses), allowing species to accumulate over larger noncontiguous landmasses over time.

What does the earliest-diverging lineage in *Arcytophyllum* and *Hedyotis* with the long lag times (~7 Ma and ~4 Ma, respectively) suggest? One common pattern is that both *Arcytophyllum thymifolum* and Thai *Hedyotis corymbiformis* group are found at both high and low altitudes (*A. thymifolium* 400–4000 m a.s.l., *H. corymbiformis* 192–1000 m a.s.l.) despite being large, woody shrubs/trees. This pattern of "delayed shifts" (see also Spriggs et al. [2014] for the C_4 photosynthetic pathway in Poaceae and associated radiations) suggests the trait (perenniality) that helped these groups to diversify may have appeared long before the group experienced rapid diversification. As indicated by the HiSSE analysis, the actual diversification might have been possible only after the "confluence" of interacting events (sensu Donoghue and Sanderson, 2015) that involved "innovation" (e.g., woody habit), environmental changes (during late Miocene, Pliocene, and uplifting of land masses creating mountains), and geographic movements (e.g., colonization of ancestral forms to Andes from other area for *Arcytophyllum*, South East Asia from tropical Africa/Madagascar for *Hedyotis*, and Hawaiian Islands from continental Asia for *Kadua*).

CONCLUSION

Spermacoceae serves as an exemplary study system for the processes involved in the evolution toward derived woody life forms. The shift from herbaceousness toward woodiness has developed multiple times under a wide array of environmental conditions, which mirrors the situation in angiosperms as a whole. While some species may have developed the woody habit in the arid habitats of Africa and Madagascar as an adaptation to drought resistance, *Arcytophyllum*, *Hedyotis*, and *Kadua* have developed their woody life form in wet habitats. For the tropical upland species of *Arcytophyllum* and *Hedyotis*, the formation of the woody habit and subsequent diversification may have occurred along the mountain orogeny subsequent to the colonization of the ancestors from a different habitat, while *Kadua* corresponds to insular woodiness radiation. While the detailed review of secondary woodiness at the angiosperm level remains ongoing (F. Lens, ongoing work), we do not have a good overview of how many angiosperm species are secondarily woody, and we do not know in which vegetation types these species are more common. Tropical islands and tropical mountain peaks are known hotspots for secondary woodiness, but also dry continental areas such as Mediterranean regions, savannahs, steppes, and (semi)deserts seem to harbor many more secondarily woody species than expected (F. Lens, ongoing work). Once this information is available, we can better investigate which environmental triggers are responsible for wood development, one of the most fundamental processes on Earth.

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