Phylogeny and Biogeography of the Plant Genus *Pachypodium* (Apocynaceae)

Background: The genus *Pachypodium* contains 21 species of succulent, generally spinescent shrubs and trees found in southern Africa and Madagascar. *Pachypodium* has diversified mostly into arid and semi-arid habitats of Madagascar, and has been cited as an example of a plant group that links the highly diverse arid-adapted floras of Africa and Madagascar. However, a lack of knowledge about phylogenetic relationships within the genus has prevented testing of this and other hypotheses about the group.

Methodology/Principal Findings: We use DNA sequence data from the nuclear ribosomal ITS and chloroplast *trn*L-F region for all 21 *Pachypodium* species to reconstruct evolutionary relationships within the genus. We compare phylogenetic results to previous taxonomic classifications and geography. Results support three infrageneric taxa from the most recent classification of *Pachypodium*, and suggest that a group of African species (*P. namaquanum*, *P. succulentum* and *P. bispinosum*) may deserve taxonomic recognition as an infrageneric taxon. However, our results do not resolve relationships among major African and Malagasy lineages of the genus.

Conclusions/Significance: We present the first molecular phylogenetic analysis of *Pachypodium*. Our work has revealed five distinct lineages, most of which correspond to groups recognized in past taxonomic classifications. Our work also suggests that there is a complex biogeographic relationship between *Pachypodium* of Africa and Madagascar.

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Introduction

Pachypodium (Apocynaceae) comprises 21 species of succulent, xerophytic shrubs and small trees distributed in Madagascar and southern Africa (Table 1). *Pachypodium* is well known for its diverse array of growth forms, from dwarf shrubs to tall monopodial 'bottle trees', as well as its showy insect-pollinated flowers (Fig. 1; Table 1; Vorster and Vorster 1973; Rauh 1985; Lavranos and Röösli 1996, 1999; Rapanarivo et al. 1999; Lüthy 2004). The center of diversity for *Pachypodium* is Madagascar, with 16 endemic species; the remaining five species are restricted to southern Africa (Fig. 1; Table 1). Most *Pachypodium* species are narrowly distributed, with specialized ecology (Vorster and Vorster 1973; Lüthy 2004; Rapanarivo et al. 1999); habitats vary from desert to subhumid grassland, although most species are restricted to extremely arid environments (i.e., 8-34 cm annual precipitation; Rapanarivo et al. 1999). Those species that occur in more mesic habitats (up to 200 cm annual precipitation; Rapanarivo et al. 1999) tend to occupy rocky outcrops that are probably edaphically arid.

The showy flowers and unusual growth forms of *Pachypodium* have made them a favorite of horticulturists, leading to the exploitation of wild plants (Lüthy 2006). Over-collecting, combined with habitat destruction (Goodman et al. 2003), has led to international trade restrictions, highlighting the need for improved systematic understanding of *Pachypodium*.

In Madagascar, *Pachypodium* forms a component of the strongly endemic xerophytic flora (Rapanarivo et al. 1999). These high levels of endemism in the xerophytic flora of Madagascar are attributed to the great antiquity of arid conditions on the island (Koechlin 1972); a climate suitable for the growth of xerophytic plants is thought to have prevailed in at least part of Madagascar throughout the Cenozoic (0-65 Ma; Wells 2003). In addition, *Pachypodium* is part

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of a large group of arid-adapted plants—including many other succulents, such as *Euphorbia* and *Aloe*—with representatives in both Africa and Madagascar (Leroy 1978; Jürgens 1997); these plants provide evidence for a biogeographic link between arid regions of Africa and Madagascar, many of which are widely disjunct from one-another or isolated by intervening mesic habitats (Leroy 1978). However, without an explicit phylogenetic framework, it is impossible to decipher the history of *Pachypodium* diversification in the Afro-Malagasy region.

Several taxonomic classifications of Malagasy *Pachypodium* have been proposed on the basis of morphological characteristics (Table 2). Nevertheless, the African species of *Pachypodium* have been inconsistently treated, leading to a lack of knowledge on their relationship to Malagasy species. Some workers have assumed that the long temporal and wide geographic separation between Madagascar and Africa (Yoder and Nowak 2006) corresponds to a deep genetic divergence between *Pachypodium* species from the two regions (Perrier de la Bâthie 1934; Lüthy 2004). Indeed, Perrier de la Bâthie (1934) suggested that the two groups might not be one-another's closest relatives. Nevertheless, the implied divergence is not strongly reflected by morphology; Lüthy (2004) cited only one trait—the presence of brachyblasts in African species—that separates the two groups. Overall, the monophyly of African and Malagasy *Pachypodium*, proposed infrageneric taxa, and *Pachypodium* itself, have never been tested.

We reconstruct the evolutionary history of *Pachypodium* using nuclear ribosomal ITS and chloroplast *trn*L-F DNA sequence data. Two additional chloroplast loci were included in the project design (*trn*S-G intergenic spacer and *rpL*16; Shaw et al. 2005), but proved insufficiently variable to justify further development. Nevertheless, both ITS and *trn*L-F have proven utility for species-level phylogenetic reconstruction in plants (Baldwin et al. 1995; Shaw et al. 2005). Our specific aims were to 1) test infrageneric classifications of *Pachypodium* and 2) determine

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relationships between the African and Malagasy members of *Pachypodium*, including patterns of diversification between the two landmasses.

Materials and Methods

Taxon sampling

We generated new ITS and *trn*L-F sequences from 56 *Pachypodium* samples representing all 27 taxa in the most recent revision of the genus (Lüthy 2004; Tables 1 and 2). An additional ITS sequence was generated for *Funtumia africana*—a close relative of *Pachypodium* (Potgieter and Albert 2001)—for use in rooting the ITS tree. *Pachypodium* and *Funtumia* tissues for DNA analysis were taken from greenhouse or garden plants (appendix 1). Tissues were obtained by D. Burge, Walter Röösli, Nicholas Plummer, and Anurag Agrawal. Specimens were identified by W. Röösli, N. Plummer, or D. Burge according to the taxonomic revision of Lüthy (2004) and subsequent descriptions of new taxa (Lüthy 2005; Lüthy and Lavranos 2005). Plants were selected based on geographic distribution, with a larger amount of sampling for widespread taxa. Between one and eight populations of each taxon were used (Tables 1 and 2). Additional non-*Pachypodium trn*L-F sequences, for rooting trees, were obtained from GenBank (*F. africana* [EF456206], *Holarrhena curtisii* [EF456122], *Kibatalia macrophylla* [EF456119], *Malouetia bequaertiana* [EF456243], and *Mascarenhasia lisianthiflora* [EF456174]). These taxa were selected on the basis of their close relationship with *Pachypodium* (Potgieter and Albert 2001).

Molecular methods

Total genomic DNA was extracted from silica-dried leaves or seeds using the DNeasy Plant Mini Kit (Qiagen, Germantown, MD) according to the manufacturer's instructions. For

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seeds, up to three excised embryos from a single parent plant were pooled prior to DNA extraction (Burge and Barker 2010). DNA was extracted from seeds when silica-dried material for the same plant was not available, or proved recalcitrant to extraction of high quality DNA. All polymerase chain reactions were performed using Qiagen *Taq* DNA Polymerase. Amplifications were performed using an initial incubation at 94°C for 10 min and 30 cycles of three-step PCR (1 min at 94°C, 30 s at 45°C, and 2 min at 72°C), followed by final extension at 72°C for 7 min. PCR was performed on a Perkin Elmer GeneAmp thermocycler. The primers ITS4 (White et al. 1990) and ITSA (Blattner and Kadereit 1999) were used to amplify the ITS1-5.8S-ITS2 region of the nuclear ribosomal DNA. Primers 'c' and 'f' (Taberlet et al. 1991) or a combination of these with internal primers 'd' and 'e' were used to amplify the *trn*L-F chloroplast region. For some plants, sequencing of ITS was problematic as a result of variation in length among ITS copies present in individual plants. As a result, cloning of the ITS region was required for some plants. Cloning was carried out using the pGEM-T Easy Vector kit (ProMega, Madison, WI) according to the manufacturer's instructions. NIA inserts were amplified directly from up to 4 positive colonies using the PCR protocol described above. For all PCR reactions, excess primer and dNTPs were removed using exonuclease I (New England Biolabs, Ipswich, MA [NEB]; 0.2 units/µl PCR product) and antarctic phosphatase (NEB; 1.0 unit/µl PCR product) incubated for 15 min at 37°C followed by 15 min at 80°C. For sequencing we used Big Dye chemistry (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. Sequences were determined bidirectionally on an Applied Biosystems 3100 Genetic Analyzer at the Duke University Institute for Genome Science and Policy Sequencing Core Facility.

Sequence editing and alignment

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All sequences were assembled and edited in Sequencher 4.1 (Gene Codes Corporation). In the case of the five plants for which ITS was cloned, we assessed sequence variation using an alignment of cloned sequences (hereafter 'isolates'). Two plants yielded pools of identical isolates (P011 and P021, appendix 1), one yielded four different types of isolate (P053), and two were represented by a single successfully cloned isolate (P046 and P048). For the plant with more than one isolate type (P053), we included all four isolates in the phylogenetic analyses of ITS; for the plants with identical isolates, we selected a single isolate to represent each plant. New ITS and *trn*L-F sequences for *Pachypodium* were deposited in GenBank (appendix 1).

The 60 new ITS and 55 new *trn*L-F sequences, along with additional outgroup sequences from GenBank, were used to create separate alignments for the two regions (Table 3; Supplemental Alignments S2 and S3). Sequences were aligned in MUSCLE (Edgar 2004) under default settings. For ITS, several indel- and repeat-rich regions (54 bp total) were excluded due to alignment ambiguity. A portion of *trn*L-F not available for some taxa (the 3' *trn*L intron) was recoded as missing data. Indels were not recoded for analysis.

Following individual alignment of ITS and *trn*L-F, we endeavored to create a combined alignment. Preliminary analyses showed that for the single *Pachypodium* sample represented by more than one cloned ITS sequence (P053; Table 1), the four sequences formed a monophyletic group. Thus, a single sequence from this group was selected at random. For the final combined alignment (Supplemental Alignment S2), the entire *trn*L-F region was coded as missing data for the two *Pachypodium* samples from which *trn*L-F was not obtained (*P. bispinosum* A049 and *P. brevicaule* subsp. *leucoxanthum*, P066). To test for conflict between the nuclear (ITS) and chloroplast (*trn*L-F) portions of the alignment, we used the incongruence length difference (ILD) test (Farris et al. 1995), implemented in PAUP* v 4.0 (Swofford 2000) as the partition

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homogeneity test. The test used 1000 random repetitions of the parsimony analysis described below (see Phylogenetic analyses). Results showed significant disagreement between ITS and *trn*L-F (P = 0.047; 953/1000 trees). To account for this conflict, we ran all of our phylogenetic analyses on the separate *trn*L-F and ITS alignments, noting any conflicts between the results, and compared these to results from the combined alignment (see Phylogenetic analyses).

Phylogenetic analyses

Trees were reconstructed using Bayesian, maximum likelihood (ML), and maximum parsimony (MP) techniques. Bayesian analyses were carried out based on the best fit model of evolution from jModelTest 2, under default parameters (Posada and Crandall 1998; Guindon and Gascuel 2003; Darriba et al. 2012; ITS: GTR+I+G; *trn*L-F: GTR+I). Bayesian sampling was performed in MrBayes v 3.0 (Ronquist and Huelsenbeck 2003). Analyses were carried out as follows: 1) three separate runs of 1 X 10^7 MCMC generations, sampling every 1000 generations, 2) examination of run output for convergence, 3) removal of the first 1000 samples (10%) as burnin after visual inspection of likelihood score plots, (4) comparison of consensus trees for each run, and (5) combination of post-burnin samples from all three runs to compute a consensus tree. A partitioned model of sequence evolution was used for the analysis of combined data.

Maximum likelihood analyses were carried out in GARLI v 2.0 (Zwickl 2006). For each alignment, two search replicates were performed in a single execution. Models of evolution were the same as those described for Bayesian analyses, with a partitioned model applied to the combined alignment. Other parameters were kept at default settings. Statistical support was inferred with 100 replicates of bootstrap reweighting (Felsenstein 1985), implemented as in the tree search.

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Maximum parsimony analysis was conducted in PAUP* v 4.0 (Swofford 2000). An initial heuristic search of 100 random taxon addition replicates was conducted with tree-bisection-reconnection branch swapping (TBR) and MULPARS in effect, retaining only ten trees from each replicate. A strict consensus of these trees was then used as a constraint tree in a second heuristic search using the similar parameters as above, but with 1000 random sequence addition replicates, and retaining 100 trees per addition replicate. We used this method due to the excessive number of trees generated by unconstrained searches. This strategy checks for shorter trees than those found by the initial search, demonstrating that the final consensus tree reflects all of the most parsimonious trees (Catalán, Kellogg, and Olmstead, 1997). We also ran searches on the three alignments using an unconstrained search with the nearest neighbor interchange (NNI) swapping algorithm, which produced trees of exactly the same length as the constrained searches. In the interest of brevity, we present results only for the constrained searches. We estimated Bootstrap support (Felsenstein 1985) for our parsimony trees using 100 pseudoreplicates and the same search setting as described above, including use of a constraint tree. We treated gaps as missing data for all phylogenetic analyses.

Results

Sequences and matrices

The ITS region had an aligned length of 658 bp (Supplemental Alignment S1). Of the 156 (included) variable positions within the ingroup, 110 were parsimony informative (Table 3). Individual *Pachypodium* ITS sequences ranged in length from 617 bp (*P. decaryi*, P009 and P011) to 632 bp (*P. rosulatum* subsp. *gracilius*, P038 and P039). Length variation was primarily

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attributable to several indel- and repeat-rich regions that could not be readily aligned, and were thus excluded from analysis, as described above.

The *trn*L-F region had an aligned length of 961 bp (Supplemental Alignment S2). Of the 33 variable positions within the ingroup, 18 were parsimony-informative (Table 3). Individual *Pachypodium trn*L-F sequences ranged in length from 747 bp (*P. lealii*, P053) to 913 bp (*P. densiflorum*, P015), though all but the *P. lealii* sequence were at least 888 bp long. The extreme shortness of the *P. lealii* sequence relative to all other sequences is due to a very large indel in the *trn*L intron (164 bp aligned length; Supplemental Alignment S2).

The combined alignment contained 61 terminals, with an aligned length of 1619 bp (Supplemental Alignment S3). Of the 184 (included) variable positions within the ingroup, 114 were parsimony informative (Table 3).

Phylogenetic trees

The Bayesian majority-rule consensus tree for ITS contained 28 resolved nodes within *Pachypodium*, including 13 with a PP of 1.0 (Supplemental Treefile S4, A; Fig. 2). By contrast, the *trn*L-F-based Bayesian tree contained 10 nodes total, including only five with a PP of 1.0 (Supplemental Treefile S5, A; Fig. 2). The combined ITS and *trn*L-F tree contained 30 nodes, including 17 with a PP of 1.0 (Supplemental Treefile S6, A; Fig. 3).

Maximum parsimony searches based on ITS data alone resulted in 4851 trees of 324 steps (Table 4; Supplemental Treefile S4, B); a total of 12 nodes had bootstrap (BS) support greater than or equal to 95% (Supplemental Treefile S4, C). Searches using *trn*L-F data alone resulted in 8 trees of 71 steps (Table 4; Supplemental Treefile S5, B); only one node had BS support greater than or equal to 95% (Supplemental Treefile S5, C). Searches on the combined

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ITS and *trn*L-F data resulted in 4582 trees of 394 steps (Table 4; Supplemental Treefile S6, B); a total of 8 nodes had BS support greater than or equal to 95% (Supplemental Treefile S6, C; Fig. 3). In all cases, use of a constraint tree failed to find any trees of equal or shorter length that contradicted the respective consensus trees (see Materials and Methods).

Maximum likelihood (ML) analyses support similar relationships to those indicated by maximum parsimony and Bayesian analyses. The best ML tree for ITS alone contained 33 resolved nodes within *Pachypodium*, including 14 with BS support greater than or equal to 95% (Supplemental Treefile S4, D & E). By contrast, the *trn*L-F-based ML tree contained 12 nodes, including only one with BS greater than or equal to 95% (Supplemental Treefile S5, D & E). The best ML tree based on ITS combined with *trn*L-F contained 33 nodes, including 10 with BS support greater than or equal to 95% (Supplemental Treefile S6, D & E; Fig. 3).

Overall, relationships recovered on the basis of *trn*L-F support those obtained from ITS, though generally at a lower level of statistical confidence. There were some cases of conflict between trees based on these two regions (Fig. 2). Here, we report on results from the combined ITS and *trn*L-F alignment (Fig. 3), which provide improved resolution in comparison to trees based on individual regions, and are generally better supported, despite containing fewer nodes with very high levels of support (PP of 1.0, or 100% support from MP or ML BS analyses).

Pachypodium is recovered as monophyletic (Fig. 3). Six of the 11 minimum-rank taxa (species & subspecies) represented by more than one sampled plant (Table 1) are monophyletic, four with very strong support (PP 1.0; MP boostrap ≥ 95%; *P. baronii*, *P. decaryi*, *P. rosulatum* subsp. *rosulatum*, and *P. windsorii*; Fig. 3). The following multi-taxon clades are also recovered with high levels of support (PP = 1.0; MP BS ≥ 95%): 1) the Malagasy *P. decaryi*, *P. rutenbergianum*, and *P. sofiense*, 2) the African *P. lealii* and *P. saundersii*, 3) the African *P.*

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namaquanum, *P. succulentum*, and *P. bispinosum*, 4) an 11-taxon group corresponding to section *Gymnopus* (Table 2), and 5) a smaller group nested within *Gymnopus* comprising *P. brevicaule* subsp. *brevicaule*, *P. densiflorum*, *P. eburneum*, *P. inopinatum*, and *P. rosulatum* subsp. *bicolor*.

Discussion

Phylogenetic relationships

Our results suggest that neither African nor Malagasy *Pachypodium* are monophyletic (Fig. 3). It was previously assumed that the long temporal and geographic isolation of Madagascar from the African continent (Yoder and Nowak 2006) implied that Malagasy and African *Pachypodium* were independent evolutionary units (e.g., Perrier de la Bâthie 1934; also see Biogeography, below). However, there is little morphological evidence to support this idea (Lüthy 2004), and our phylogenetic analyses do not provide evidence for the monophyly of African or Malagasy *Pachypodium* (Fig. 3).

Additionally, our exhaustive sampling of *Pachypodium* species and subspecies (Table 1) has provided the opportunity to test existing morphology-based hypotheses on infrageneric relationships. Our results support the most recent infrageneric classification of *Pachypodium* proposed by Lüthy (2004; Table 2). Lüthy's (2004) shrubby, predominantly yellow-flowered section *Gymnopus* is clearly monophyletic (Fig. 3, PP 1.0; MP & ML BS 100%), as is the shrubby, red-flowered section *Porphyropodium* (Fig. 3, PP 0.98; MP and ML 98%). Our results also indicate a very close relationship between *Porphyropodium* and *Gymnopus* (Fig. 3, PP 0.96; MP and ML BS > 86%), a relationship not emphasized by past classifications. Finally, the third section recognized in Lüthy's (2004) classification, the mostly arborescent, white-flowered *Leucopodium*, is marginally supported in the combined phylogenetic tree (Fig. 3, PP 0.94; ML

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BS 71%). Overall, our results also support the tradition of using corolla color as a basis for circumscription of taxa within *Pachypodium* (Fig. 3; Poisson 1924; Pichon 1949; Perrier de la Bâthie 1934; Lüthy 2004). Nonetheless, we agree with Lüthy (2004), that an ideal infrageneric classification should use multiple morphological characteristics to define groups.

Below the section level, previous classifications of *Pachypodium* are not well supported by our molecular phylogenetic results. One clear exception is Lüthy's (2004) series Contorta (Table 2), which was defined on the basis of seed morphology to include the arborescent *P. rutenbergianum* and *P. sofiense*, as well as the limestone-endemic *P. decaryi*. Our results show that this group is strongly monophyletic (Fig. 3, PP 1.0; MP and ML BS 100%), confirming the detailed work of Lüthy (2004) but contrasting with most previous opinions, for example Pichon (1949), who allied *P. decaryi* with another limestone endemic, *P. ambongense*. Within section *Gymnopus*, Lüthy's (2004) series *Densiflora* (Table 2) roughly corresponds to a clade that we recover nested inside *Gymnopus* (Fig. 3, Clade A, PP 1.0; MP BS 76%). However, Clade A includes *P. rosulatum* subsp. *bicolor* and *P. brevicaule* subsp. *brevicaule*, both considered members of series *Ramosa* by Lüthy (2004). Our results indicate that the floral characters used by Lüthy (2004) and others to define groups within *Gymnopus* (Table 2) are homoplasious.

Most past classifications of *Pachypodium* have dealt in very sparse detail, if at all, with the distinctive and morphologically heterogeneous African members of the genus. As outlined above, our results indicate that African *Pachypodium* are not monophyletic. Overall, the molecular phylogenetic results show that African *Pachypodium* are divided into two very distinctive lineages, one comprising the morphologically similar *P. lealii* and *P. saundersii* (Rapanarivo et al. 1999), and a second containing the bizarre monopodial tree *P. namaquanum* and the tuberous shrubs *P. bispinosum* and *P. succulentum*. The close relationship between *P.*

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lealii and *P. saundersii* (Fig. 3, PP 1.0; MP BS 95%) has been noted for some time, as indicated by a reduction to synonymy under *P. saundersii* that was undertaken by Rowley (1973). The close relationship of *P. namaquanum* to *P. bispinosum* and *P. succulentum* was less expected (Fig. 3, PP 1.0; MP and ML BS 100%). However, Vorster and Vorster (1973) proposed a close relationship between *P. namaquanum* and *P. bispinosum* based on corolla shape. These authors also proposed that the asymmetrical flowers of *P. succulentum* linked this species to *P. lealii* and *P. saundersii* more than to *P. bispinosum*. Our results clearly show that *P. bispinosum* and *P. succulentum* are one another's closest relatives, sister to *P. namaquanum*.

Additional molecular phylogenetic work will be required to obtain better support for basal-branching relationships in *Pachypodium*, particularly the relationship between African and Malagasy species. This work will likely require the sequencing of additional loci, from both the chloroplast and nuclear genome. Resolution of relationships among species from Lüthy's (2004) section *Gymnopus* will also require additional work. For this group, rapidly evolving genetic markers such as AFLPs and microsatellites might yield more insight into relationships.

Biogeography

A recent estimate of 37-64 Ma for the divergence of stem-lineage Apocynaceae from its closest relatives among the Gentianales (Bell et al. 2010) implies that the age of origin for *Pachypodium* is probably more recent than the ~ 80 Ma timing for the ultimate isolation of Madagascar from other continental areas (Yoder and Nowak 2006). In fact, a recent review of Madagascar biogeography suggests that most of Madagascar's biotic connections are best explained by long-distance dispersal during the Cenozoic rather than ancient Gondwanan vicariance (Yoder and Nowak 2006). Thus, if *Pachypodium* did not originate in Madagascar it

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must have arrived on the island via long-distance dispersal. However, our phylogenetic reconstructions lack resolution among major African and Malagasy lineages of *Pachypodium* (Figs. 2 & 3), preventing reliable reconstruction of geographic range evolution, including dispersal-vicariance scenarios between Africa and Madagascar.

Nonetheless, within Madagascar, several large-scale patterns of diversification are discernable when comparing phylogenetic results to information on the geographic distributions and ecology of the taxa. Within section *Gymnopus*, a clade of predominantly high-elevation taxa is discernable (Fig. 3, Clade A; Rapanarivo et al. 1999); five out of the six taxa in this group are known to occur above 1400 m (Table 1). Most members of Clade A are found exclusively on bare-rock areas within the subhumid forest biome of the central highlands (inselbergs; Guillamet 1984; Barthlott and Porembski 1996; Fig. 4). This is in contrast to remaining *Gymnopus*, which tend to occur at lower elevations in the dry deciduous forest, succulent woodland, and spiny thicket biomes, where they also specialize on various kinds of rocky habitats (Table 1; Fig. 4). Diversification of Clade A has occurred almost entirely on inselbergs of the central highlands and is characterized by very low levels of sequence divergence at the ITS and *trn*L-F loci (Figs. 2 & 3). One possible explanation for the phylogenetic and ecological isolation of this group is the expansion of forests and other moisture-loving plant communities in Madagascar during the late Cenozoic, which probably led to the gradual isolation of previously widespread xerophytes into ecologically suitable areas (Wells 2003). Such isolation may have led to the high levels of endemism now seen in the unique and highly celebrated xerophytic plant communities of Madagascar's inselbergs (Guillamet 1984; Barthlott and Porembski 1996; Wells 2003). Overall, adaptation to high elevations by Malagasy *Pachypodium* is all but restricted to members of

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Gymnopus and the closely allied *Porphyropodium* (Table 1; Fig. 3). Outside this group, only one species of Malagasy *Pachypodium*, *P. lamerei*, is known to occur above 1000 m (Table 1).

Lower-elevation Malagasy *Pachypodium* are mostly members of *Leucopodium*, a group of arborescent taxa that is associated with the dry deciduous forests, succulent woodlands, and spiny thickets (Fig. 4B). The large stature of most *Leucopodium* may represent an adaptation to the forest habitat; shrub-like members of the group, *P. decaryi*, *P. menabeum*, and *P. ambongense*, are all endemics on limestone outcrops at moderate elevations (Lüthy 2006), a setting in which competition for light may be less intense than in densely forested habitats.

Conservation

Conservation planning for threatened flora and fauna must take into consideration the evolutionary potential of populations and taxa (Forest et al. 2007). Ignoring evolutionary potential in long-term planning may lead to losses of diversity that compromise the ability of these groups to adapt and thus survive in the long-term, particularly in response to ongoing global climate change. Thus, it is important to consider the phylogenetic and population-genetic diversity of a group when planning for its conservation. In the case of *Pachypodium*, phylogenetic results presented here show that several species and groups of species are strongly divergent from other *Pachypodium* (e.g., *P. decaryi* and most African *Pachypodium;* Fig. 3). These groups represent important islands of phylogenetic diversity within *Pachypodium*, the loss of which would drastically reduce the overall diversity of the genus. Many members of the *Gymnopus* section of *Pachypodium*, by contrast, are very shallowly divergent based on our results (Fig. 3). The members of *Gymnopus* are adapted to a great variety of habitats, and therefore may contain much ecological diversity in terms of local adaptation (Lüthy 2004).

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However, in comparison to highly divergent taxa such as *P. decaryi*, each individual *Gymnopus* taxon represent a very small proportion of the total phylogenetic diversity of *Pachypodium*. In light of the always-limited resources available for conservation, an effort should be made to prioritize the protection of phylogenetically divergent lineages of *Pachypodium* as well as the overall genetic diversity of the genus. We recommend stronger conservation measures—including greater restrictions on the trade of wild-collected plants—for very narrowly distributed species having Bayesian PP of 1.0 in the combined ITS and *trn*L-F tree (Fig. 3). This includes the Malagasy *P. baronii*, *P. windsorii*, and *P. decaryi*. The highly divergent African species are not included in this list due to their relatively wide geographic distributions.

Acknowledgments

For tissue samples we thank Ralph Hoffmann, Nicholas Plummer, Walter Röösli, and the National Botanic Garden of Belgium, with logistical assistance from Frank Van Caekenberghe. Comments on drafts and data interpretation were provided by Jonas Lüthy, Nicholas Plummer, and Katherine Zhukovsky. Sketches of *Pachypodium* were rendered by Bonnie McGill.

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Appendix 1. Sampled plants and DNA sequences. For each plant the within-study code is in brackets, followed by collector and collector number, herbarium or living collection for deposition of voucher specimen (in parentheses; ZSS indicates living collection of Sukkulenten-Sammlung Zürich), provenance, and GenBank numbers for ITS and *trn*L-F; Abbreviation 's.n.' indicates no collection number.

Funtumia africana—[OG1] National Botanic Garden of Belgium 19514728 (BR), cultivated Plant; ITS: KC189049.

Pachypodium ambongense—[P003] W. Röösli, R. Hoffman, & M. Grubenmann, s.n., collected 25.xi.1989 (P, ZSS), Namoroka, Madagascar; ITS: HQ847410; *trn*L-F: HQ847465. ***P. baronii***—[P004] A. Razafindratsira, s.n., collected 3.i.1988 (ZSS), Befandriana Nord, Madagascar; ITS: HQ847411; *trn*L-F: HQ847466. [P005] W. Röösli & B. Rechberger, s.n., collected xii.1990 (ZSS), Mandritsara, Madagascar; ITS: HQ847412; *trn*L-F: HQ847467. ***P. bispinosum***—[A049] A. Agrawal, s.n. (DUKE), cultivated plant; ITS: JN256214. ***P. brevicaule*** **subsp.** ***brevicaule***—[P006] W. Röösli & R. Hoffman 92/98 (ZSS), Mount Ibity, Madagascar; ITS: HQ847414; *trn*L-F: HQ847469. [P007] W. Röösli & R. Hoffman 43/01 (Z), Ranomainty, Madagascar; ITS: HQ847415; *trn*L-F: HQ847470. [P008] J. Lüthy, s.n., collected 1.vi.2006 (ZSS), Andrembesoa, Madagascar; ITS: HQ847416; *trn*L-F: HQ847471. ***P. brevicaule*** **subsp.** ***leucoxanthum***—[P066] J. Lüthy, s.n., collected 6.i.2006 (ZSS), undisclosed locality, Madagascar; ITS: KC189050.***P. decaryi***—[P009] W. Rauh 72255 (HEID), Montagne des Francais, Madagascar; ITS: HQ847417; *trn*L-F: HQ847472. [P010] W. Röösli & R. Hoffman 22/99 (ZSS), Montagne des Francais, Madagascar; ITS: HQ847418; *trn*L-F: HQ847473. [P011] W. Röösli & R. Hoffman 22/00 (ZSS), Ankarana, Madagascar; ITS: HQ847419; *trn*L-F:

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HQ847474. ***P. densiflorum***—[P012] W. Röösli & R. Hoffman 01/94 (ZSS), Mount Ibity, Madagascar; ITS: HQ847420; *trn*L-F: HQ847475. [P013] W. Röösli & R. Hoffman 42/01 (ZSS), Ranomainty, Madagascar; ITS: HQ847421; *trn*L-F: HQ847476. [P014] W. Röösli & R. Hoffman, s.n., collected 1.xii.1992 (ZSS), Ambatofinandrahana, Madagascar; ITS: HQ847422; *trn*L-F: HQ847477. [P015] W. Röösli & B. Rechberger, s.n., collected 20.i.1989 (ZSS), Fianarantsoa, Madagascar; ITS: HQ847423; *trn*L-F: HQ847478. [P016] W. Röösli & R. Hoffman 57/98 (K, P, WAG), Plateaux Horombe, Madagascar; ITS: HQ847424; *trn*L-F: HQ847479. [P017] W. Röösli & R. Hoffman 45/93 (ZSS), 107 km W Antsirabe, Madagascar; ITS: HQ847425; *trn*L-F: HQ847480. [P018] W. Röösli & R. Hoffman 31/03 (ZSS), Mahatsinjo, Madagascar; ITS: HQ847426; *trn*L-F: HQ847481. [P049] A. Razafindratsira, s.n., collected xii.2006 (ZSS), Ambodiriana, Madagascar; ITS: HQ847427; *trn*L-F: HQ847482. ***P. eburneum***—[P019] W. Röösli & R. Hoffman 01/96 (P, MO, TAN, WAG, ZSS), Mount Ibity, Madagascar; ITS: HQ847428; *trn*L-F: HQ847483. [P020] J. Lüthy, s.n., collected 1.vi.2006 (ZSS), Andrembesoa, Madagascar; ITS: HQ847429; *trn*L-F: HQ847484. ***P. geayi***—[P021] W. Röösli & R. Hoffman 29/04 (ZSS), Ifaty, Madagascar; ITS: HQ847430; *trn*L-F: HQ847485. ***P. horombense***—[P022] W. Röösli & B. Rechberger, s.n., collected 21.xii.1990 (ZSS), Betroka, Madagascar; ITS: HQ847431; *trn*L-F: HQ847486. [P023] W. Röösli & R. Hoffman 34/01 (ZSS), Beraketa, Madagascar; ITS: HQ847432; *trn*L-F: HQ847487. [P024] W. Röösli & R. Hoffman 73/96 (WAG), Andalatanosy, Madagascar; ITS: HQ847433; *trn*L-F: HQ847488. ***P. inopinatum***—[P025] W. Röösli & R. Hoffman 46/93 (P, TAN, HEID, WAG, ZSS), Manakana, Madagascar; ITS: HQ847434; *trn*L-F: HQ847489. ***P. lamerei***—[P001] W. Röösli & R. Hoffman 18/06 (ZSS), Fiherenana River, Madagascar; ITS: HQ847435; *trn*L-F: HQ847490. [P026] W. Röösli & R. Hoffman 20/02 (ZSS), Fiherenana River, Madagascar; ITS: HQ847436; *trn*L-F: HQ847491.

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[P027] W. Röösli & R. Hoffman, s.n., collected 26.i.1994 (WAG, ZSS), Ihosy, Madagascar; ITS: HQ847437; *trn*L-F: HQ847492. [P028] W. Röösli & R. Hoffman, s.n., collected 24.i.1994 (ZSS), Beraketa, Madagascar; ITS: HQ847438; *trn*L-F: HQ847493. [P029] W. Röösli & R. Hoffman 31/01 (WAG, ZSS), Andalatanosy, Madagascar; ITS: HQ847439; *trn*L-F: HQ847494. [P030] W. Röösli & R. Hoffman 19/01 (ZSS), Lac Anony, Madagascar; ITS: HQ847440; *trn*L-F: HQ847495. [P031] W. Röösli & R. Hoffman 79/96 (P, WAG, ZSS), Fort Dauphin, Madagascar; ITS: HQ847441; *trn*L-F: HQ847496. ***P. lealii***—[P053] Huntington Botanic Garden 85642 (DUKE), cultivated Plant; ITS: HQ847442; JN256217; JN256216; JN256215; *trn*L-F: HQ847497. ***P. menabeum***—[P032] W. Röösli & B. Rechberger, s.n., collected 10.xii.1991 (ZSS), Antsalova, Madagascar; ITS: HQ847443; *trn*L-F: HQ847498. [P033] W. Röösli & R. Hoffman 07/03 (ZSS), Antsalova, Madagascar; ITS: HQ847444; *trn*L-F: HQ847499. [P034] W. Röösli & R. Hoffman 03/02 (ZSS), Bekopaka, Madagascar; ITS: HQ847445; *trn*L-F: HQ847500. ***P. mikea***—[P002] W. Röösli & R. Hoffman 26/05 (P, TAN), South of Morombe, Madagascar; ITS: HQ847446; *trn*L-F: HQ847501. ***P. namaquanum***—[P054] J. Lüthy, s.n. (University of Bern Institute of Plant Sciences, *living collection), cultivated Plant; ITS: HQ847447; trnL-F: HQ847502.* ***P. rosulatum* subsp. *bemarahense***—[P035] W. Röösli & R. Hoffman 08/03 (TAN), Antsalova, Madagascar; ITS: HQ847448; *trn*L-F: HQ847503. ***P. rosulatum* subsp. *bicolor***—[P036] W. Röösli & R. Hoffman 42/93 (P, MO, TAN, WAG, ZSS), Berevo, Madagascar; ITS: HQ847449; *trn*L-F: HQ847504. ***P. rosulatum* subsp. *cactipes***—[P037] W. Röösli & R. Hoffman 77/96 (BR, K, MO, P, TAN, WAG, ZSS), Fort Dauphin, Madagascar; ITS: HQ847450; *trn*L-F: HQ847505. ***P. rosulatum* subsp. *gracilius***—[P038] W. Röösli & R. Hoffman 36/01 (ZSS), Isalo, Madagascar; ITS: HQ847451; *trn*L-F: HQ847506. [P039] W. Röösli & R. Hoffman 42/05 (K, MO, WAG), Bezaha, Madagascar; ITS: HQ847452;

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*trn*L-F: HQ847507. ***P. rosulatum* subsp. *makayense***—[P040] W. Röösli & R. Hoffman 08/02 (MO, P, TAN), Makay, Madagascar; ITS: HQ847453; *trn*L-F: HQ847508. ***P. rosulatum* subsp. *rosulatum***—[P041] W. Röösli & R. Hoffman 26/96 (WAG, ZSS), Antsakabary, Madagascar; ITS: HQ847454; *trn*L-F: HQ847509. [P042] W. Röösli & R. Hoffman 21/95 (MO, P, WAG, ZSS), Mandritsara, Madagascar; ITS: HQ847455; *trn*L-F: HQ847510. [P043] A. Razafindratsira, s.n., collected 30.xii.1991 (ZSS), Bealanana, Madagascar; ITS: HQ847456; *trn*L-F: HQ847511. [P044] W. Röösli & R. Hoffman 29/95 (ZSS), Ananalava, Madagascar; ITS: HQ847457; *trn*L-F: HQ847512. [P045] W. Röösli & R. Hoffman 23/03 (ZSS), Benetsy, Madagascar; ITS: HQ847458; *trn*L-F: HQ847513. ***P. rutenbergianum***—[P046] W. Röösli & R. Hoffman 19a/95 (ZSS), Anjohibe, Madagascar; ITS: HQ847459; *trn*L-F: HQ847514. ***P. saundersii***—[P055] M. Lehmann, s.n. (plants grown by N. Plummer) (DUKE), Karongwe Game Reserve, South Africa; ITS: HQ847460; *trn*L-F: HQ847515. ***P. sofiense***—[P048] W. Röösli & R. Hoffman 14/96 (P, WAG), Mandritsara, Madagascar; ITS: HQ847461; *trn*L-F: HQ847516. ***P. succulentum***—[P056] J. Lavranos, s.n. (University of Bern Institute of Plant Sciences, living collection), Grahamstoon, South Africa; ITS: HQ847462; *trn*L-F: HQ847517. ***P. windsorii***—[P050] A. Razafindratsira, s.n., collected 22.xii.1989 (ZSS), Windsor Castle, Madagascar; ITS: HQ847463; *trn*L-F: HQ847518. [P051] W. Röösli & R. Hoffman 17/00 (ZSS), Montagne des Francais, Madagascar; ITS: HQ847464; *trn*L-F: HQ847519.

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Table 1(on next page)

Pachypodium species, sampling, geography, and traits.

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Taxon	Sampled	Geography	Form	Corolla	Elevation
Pachypodium ambongense H.Poiss.	1	Madagascar	Shrub	White	0-100
P. baronii Constantin and Bois	2	Madagascar	Shrub	Red	300-1200
P. bispinosum (L.f.) A.DC.	1	Southern Africa	Shrub	Pink	20-800
P. brevicaule Baker subsp. *brevicaule*	3	Madagascar	Shrub	Yellow	1300-1900
P. brevicaule Baker subsp. *leucoxanthum* Lüthy	1	Madagascar	Shrub	White	Unknown
P. decaryi H.Poiss.	3	Madagascar	Shrub	White	30-350
P. densiflorum Baker	8	Madagascar	Shrub	Yellow	200-1750
P. eburneum Lavranos and Rapan.	2	Madagascar	Shrub	White	1700
P. geayi Costantin and Bois	1	Madagascar	Tree	White	2-300
P. horombense H.Poiss.	3	Madagascar	Shrub	Yellow	400-1100
P. inopinatum Lavranos	1	Madagascar	Shrub	White	1450
P. lamerei Drake	7	Madagascar	Tree	White	10-1200
P. lealii Welw.	1	Southern Africa	Tree	White	50-1600
P. menabeum Leandri	3	Madagascar	Tree	White	300-900
P. mikea Lüthy	1	Madagascar	Tree	White	2-300
P. namaquanum (Wyley ex Harv.) Welw.	1	Southern Africa	Shrub	Red	300-900
P. rosulatum Baker subsp. *bemarahense* Lüthy and Lavranos	1	Madagascar	Shrub	Yellow	300-900
P. rosulatum Baker subsp. *bicolor* (Lavranos and Rapan.) Lüthy	1	Madagascar	Shrub	Yellow	30
P. rosulatum Baker subsp. *cactipes* (K.Schum.) Lüthy	1	Madagascar	Shru	Yellow	2-1800

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			b		
P. rosulatum Baker subsp. *gracilius* (H.Perrier) Lüthy	2	Madagascar	Shrub	Yellow	300-1000
P. rosulatum Baker subsp. *makayense* (Lavranos) Lüthy	1	Madagascar	Shrub	Yellow	650
P. rosulatum Baker subsp. *rosulatum*	5	Madagascar	Shrub	Yellow	100-600
P. rutenbergianum Vatke	1	Madagascar	Tree	White	40-700
P. saundersii N.E.Br.	1	Southern Africa	Shrub	White	70-1200
P. sofiense (H.Poiss.) H.Perrier	1	Madagascar	Tree	White	20-600
P. succulentum (L.f.) A.DC.	1	Southern Africa	Shrub	Pink	50-1400
P. windsorii H.Poiss.	2	Madagascar	Shrub	Red	270-390

Note. *Taxon*, according to revision of Lüthy 2004; *Sampled*, number of individuals sampled for genetic analysis; *Geography*, indicates whether the species is endemic to Madagascar or southern Africa; *Corolla*, indicates the overall color of the corolla. *Elevation*, gives the range of elevation (m) over which the taxon is found (Rapanarivo et al. 1999; Lüthy 2006).

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Table 2(on next page)

Summary of *Pachypodium* classification.

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Subgenus	Section	Series	Species or subspecies
Nesopodium	*Gymnopus*	*Ramosa*	*P. brevicaule* subsp. *brevicaule*
			P. brevicaule subsp. *leucoxanthum*
			P. rosulatum subsp. *bemarahense*
			P. rosulatum subsp. *bicolor*
			P. rosulatum subsp. *cactipes*
			P. rosulatum subsp. *gracilius*
			P. rosulatum subsp. *makayense*
			P. rosulatum subsp. *rosulatum*
		Densiflora	*P. densiflorum*
			P. eburneum
			P. horombense
			P. inopinatum
	Leucopodium	*Contorta*	*P. decaryi*
			P. rutenbergianum
			P. sofiense
		Ternata	*P. geayi*
			P. lamerei
			P. mikea
		Pseudoternata	*P. ambongense*
			P. menabeum
	Porphyropodium		*P. baronii*
			P. windsorii
Pachypodium			*P. bispinosum*
			P. lealii
			P. namaquanum
			P. saundersii
			P. succulentum

Note. See Table 1 for taxon authorities; table includes later descriptions of new *Pachypodium* species by Lüthy (2005; *P. mikea*), Lüthy and Lavranos (2005; *P. rosulatum* subsp. *bemarahense*), and Lüthy (2008; *P. brevicaule* subsp. *leucoxanthum*).

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Table 3(on next page)

Summary statistics for DNA alignments.

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Name	Region	Terminals	Total length	Included length	G + C	Variable	PIC
Supplemental Alignment S1	ITS	60	658	604	53.7 %	156 (226)	110 (116)
Supplemental Alignment S2	*trn*L-F	59	961	961	36.4 %	33 (64)	18 (36)
Supplemental Alignment S3	ITS and *trn*L-F	61	1619	1565	43.1 %	184 (285)	114 (140)

Note. *Total Length*, the length of the complete alignment, counting portions excluded from analysis; *Included length*, the total number of characters included in the phylogenetic analysis. *G + C*, the G + C content of the complete (total length) alignment; *Variable*, the number of variable characters in the ingroup, followed by the number of variable characters in the full alignment (in parentheses); *PIC*, the number of parsimony-informative characters in the ingroup, followed by the number of parsimony informative characters in the full alignment (in parentheses).

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Table 4(on next page)

Summary statistics for maximum parsimony tree searches.

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Tree	Region	Total MP trees	Steps	CI	RI
Supplemental Treefile S4, B	ITS	4851	324	0.82	0.95
Supplemental Treefile S5, B	*trn*L-F	8	71	0.93	0.97
Supplemental Treefile S6, B	ITS and *trn*L-F	4582	394	0.83	0.92

Note. *CI*, consistency index; *RI*, retention index.

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Figure 1

Geographic distribution of *Pachypodium*

Inset is sampling of *Pachypodium* in Madagascar (appendix 1). Sampling in Africa not mapped. Data for distributions is approximate, adapted from Lüthy (2006) and Vorster and Vorster (1973).

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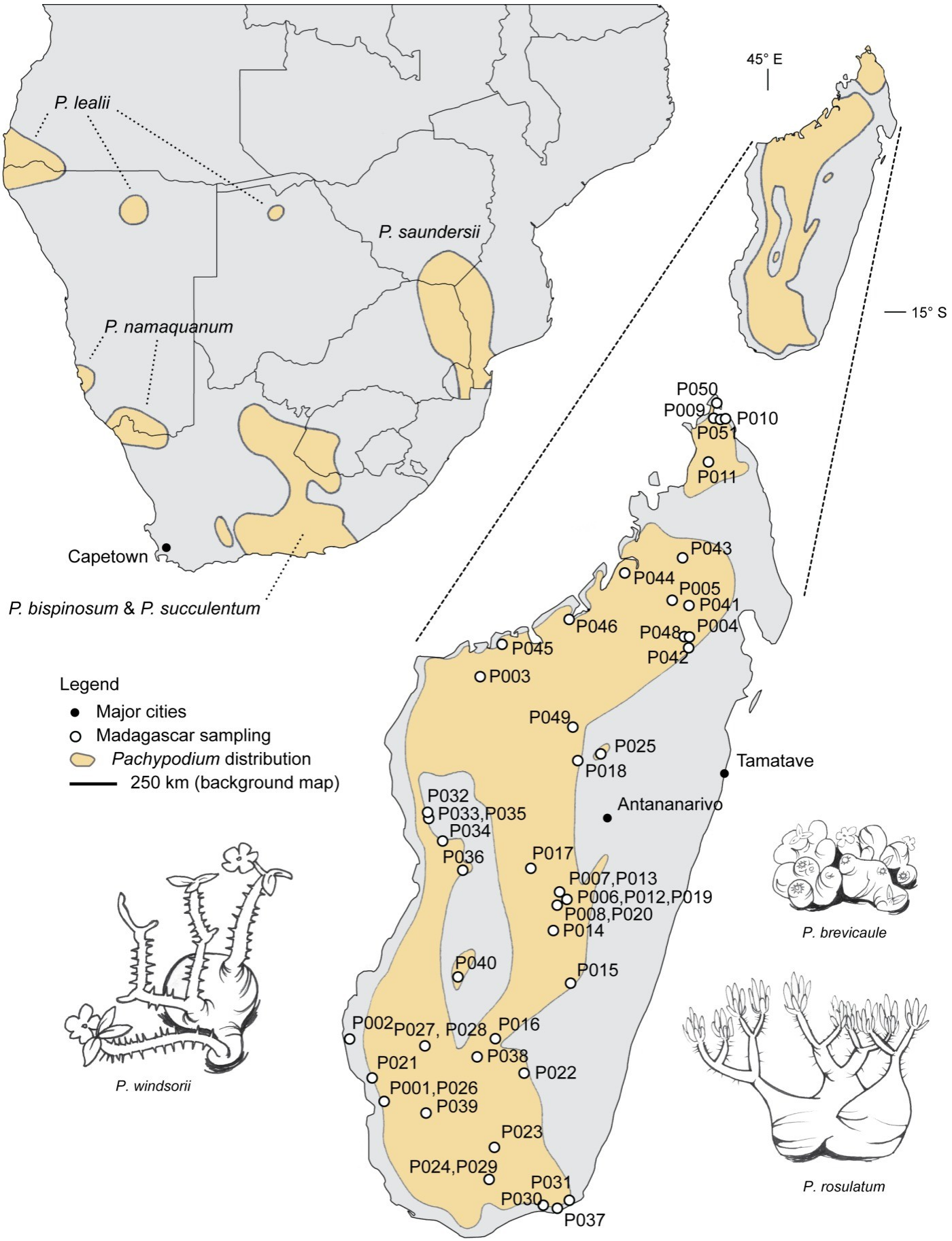

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Figure 2

Geography of *Pachypodium* in Madagascar

A, terrestrial ecoregions of Madagascar (Olson et al. 2001). B-D, Geographic distribution of major infrageneric taxa (Lüthy [2004; 2006]; Fig. 1). B, *Leucopodium*; C, *Porphyropodium*; D, *Gymnopus*.

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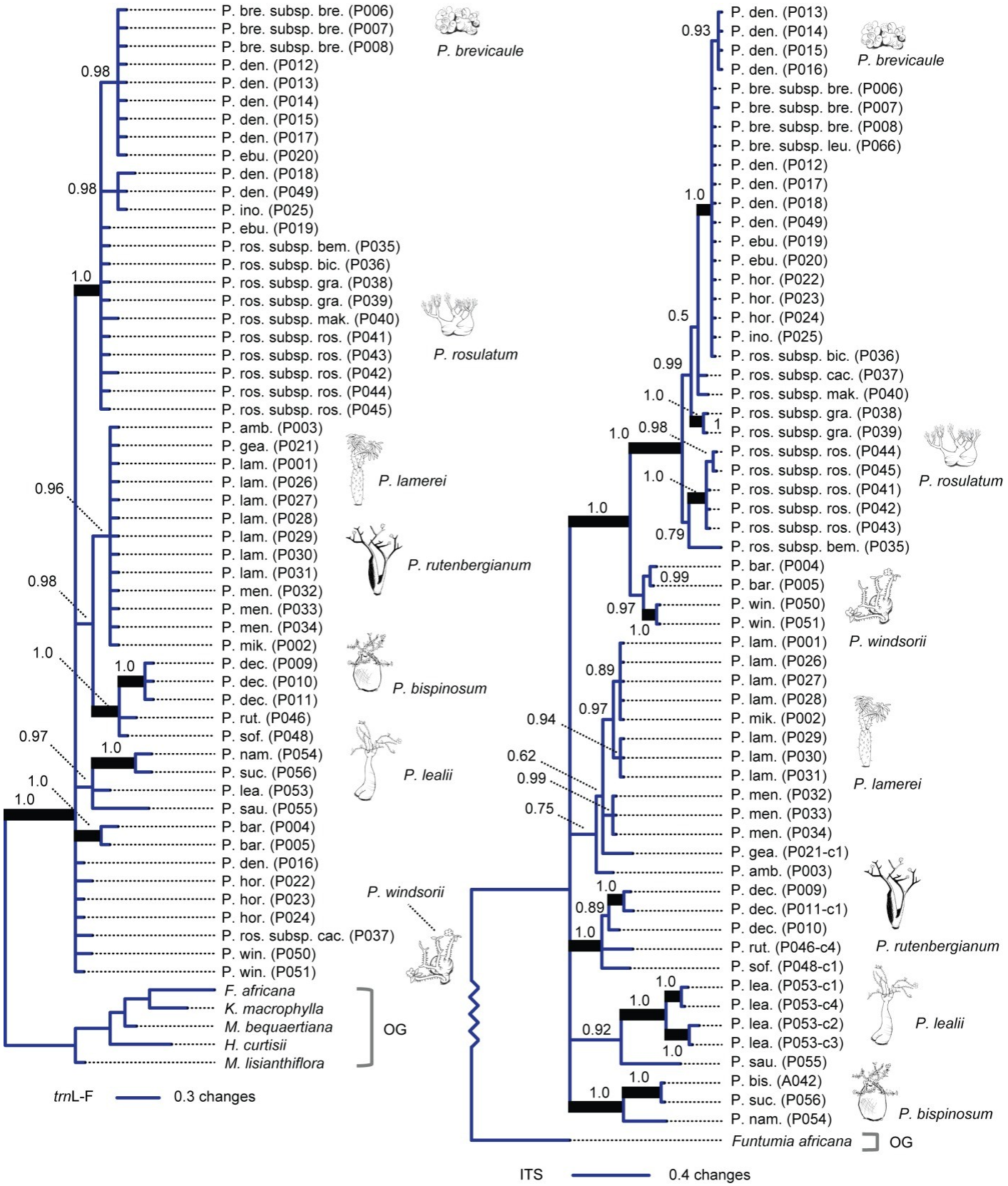

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Figure 3

Bayesian consensus phylograms for individual genetic regions

Numbers above branches are Bayesian posterior probability (PP); thickened branches have PP of 1.0. Taxon names are abbreviated (Table 1). Support values not shown for relationships among outgroups (see Supplemental Treefile S4 and S5). Zigzag line indicates a branch not shown to scale (see Supplemental Treefile S4 and S5).

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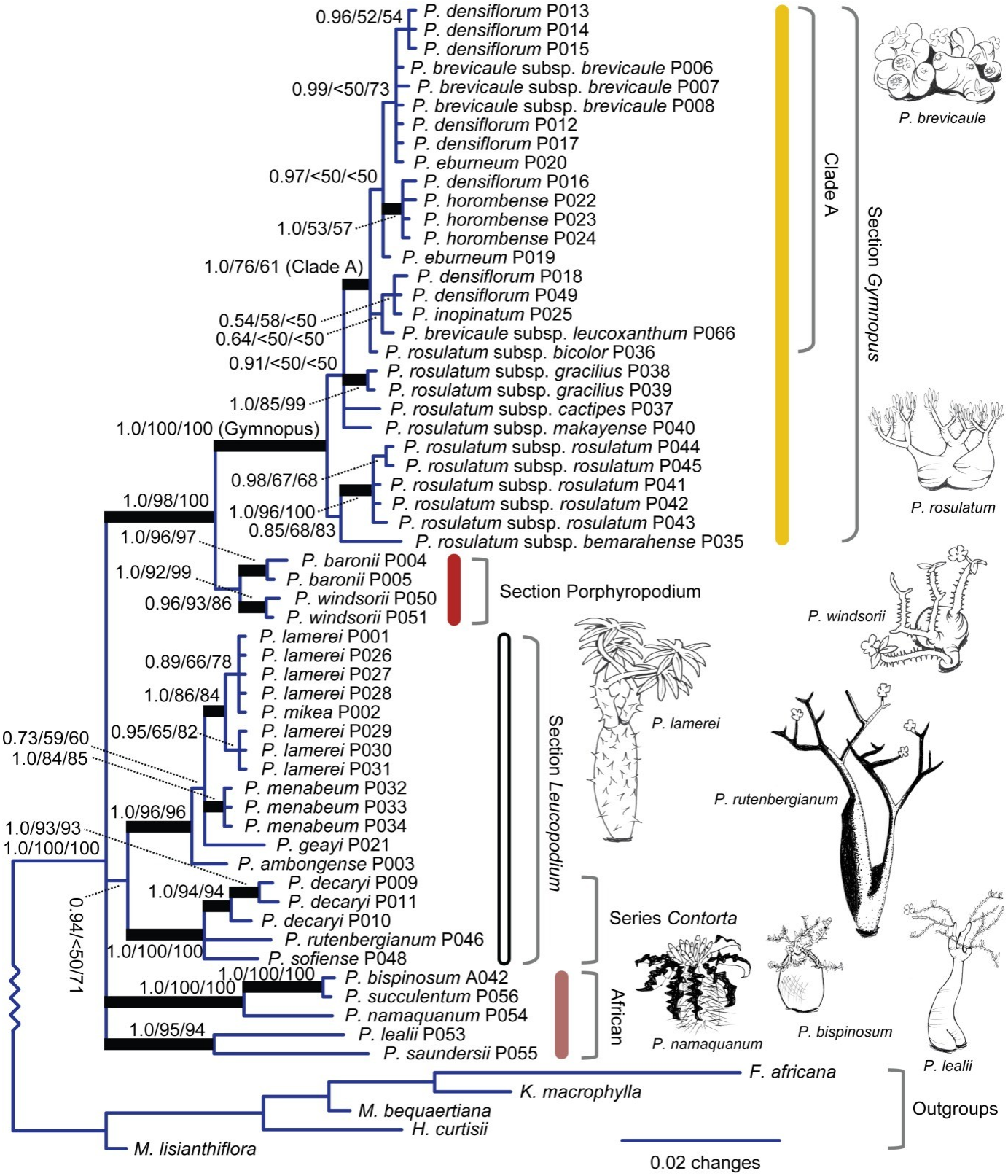

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Figure 4

Bayesian consensus phylogram for combined data

Numbers above branches are (from left to right) 1) Bayesian posterior probability (PP), 2) maximum parsimony bootstrap support, and 3) maximum likelihood bootstrap support; thickened branches have PP of 1.0. Selected subgeneric taxa are from of Lüthy (2004); colored bars indicate predominant color of corolla lobes (Table 1). Support values not shown for relationships among outgroups (see Supplemental Treefile S6). Zigzag line indicates a branch not shown to scale (see Supplemental Treefile S6).

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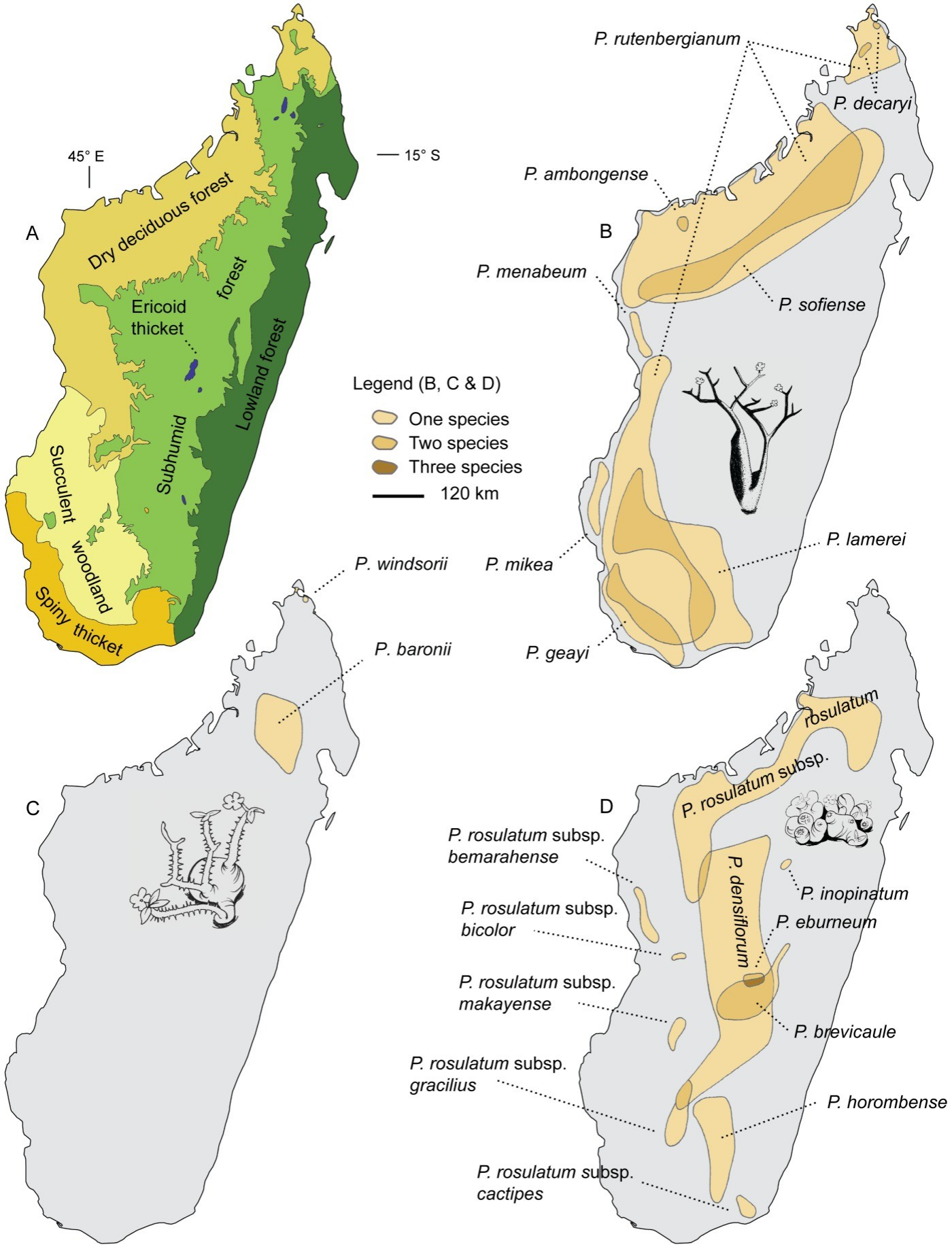

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