other name: molecular systematics and the taxonomy of *Podocarpus* and the Podocarpaceae in southern Africa

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We briefly review the taxonomic history of the Podocarpaceae, with an emphasis on the recognition of numerous segregate genera out of *Podocarpus sensu lato*. Despite some controversy over the recognition of these genera, molecular data (DNA sequences) provide evidence that supports this taxonomy. The implications for African Podocarpaceae are discussed. In particular, molecular data support the recognition of *Afrocarpus* as distinct from *Podocarpus*. Additional taxonomic problems concerning the possible segregation of *Podocarpus milanjianus* from *P. latifolius* are addressed using DNA sequence data from the nuclear internal transcribed spacer 2 (ITS2) region. Results of this are inconclusive, and suggest that alternative DNA-based evidence, such as from AFLPs or microsatellites, may be more informative in resolving such species complexes in African *Podocarpus*.

Introduction

The Podocarpaceae is the most diverse family of conifers, both morphologically and ecologically.[1] This family is concentrated in the southern hemisphere, with most of the generic level diversity being found in New Caledonia, New Zealand and Tasmania. At the species level, large numbers of species of *Podocarpus* occur in both South America and the Indonesian islands, the latter also *Dacrydium* Sol. ex Forst. are the largest (*Podocarpus* having 107 species according to Farjon[2]) and account for most of the diversity in the Podocarpaceae.[1] A number of genera (but no species) are shared between New Zealand and South America, suggesting that this family once had a wide distribution across southern Gondwanaland. The various genera within the Podocarpaceae adapted and evolved once this land mass split.[1]

Generic classification of the Podocarpaceae

Until 1970, the Podocarpaceae were viewed as comprising seven genera (*Podocarpus, Dacrydium, Phyllocladus* Rich. ex Mirb., *Acmopyle* Pilg., *Microcachrys* Hook. f., *Saxegothaea* Lindl. and *Pherosphaera* W. Archer bis (= *Microstrobos* J. Garden & L.A.S. Johnson, nom. inval.: Brummitt *et al.*[3]). All African taxa were placed in *Podocarpus*, as was accepted by Leistner[4] in his *Flora of Southern Africa* (FSA) treatment of the four southern African species of *Podocarpus* (viz. *P. falcatus* Thunb., *P. elongatus* (Sol.) L'Hér. ex Pers., *P. henkelii* Stapf ex Dallim. & A. B. Jacks., and *P. latifolius* (Thunb.) R. Br. ex Mirb.).

Podocarpus had been subdivided into eight sections on the basis of leaf anatomy[5] (sections *Afrocarpus* J. Buchholz & N. E. Gray, *Dacrycarpus* Endl., *Eupodocarpus* Endl., *Microcarpus* Pilg., *Nageia* (Gaertn.) Endl., *Polypodiopsis* C.E. Bertrand (non *Polypodiopsis* Carriére nom. rej. prop.[6]), *Stachycarpus* Endl. and *Sundacarpus* J. Buchholz & N.E. Gray). Using new data on embryology, gametophyte development, female cone structure and cytology, Quinn[7] concluded that these eight sections of *Podocarpus* should be raised to generic rank but did not make any formal proposals. A year earlier, de Laubenfels[8] had also reached similar conclusions and elevated section *Dacrycarpus* to generic rank as *Dacrycarpus* (Endl.) de Laub. and sections *Nageia, Polypodiopsis* and *Afrocarpus* together as the new genus *Decussocarpus* de Laub., an illegitimate name (the earliest name being *Nageia* Gaertn.) Three years later, de Laubenfels[9] raised section *Microcarpus* to generic rank as the monotypic genus *Parasitaxus* de Laub., while he resurrected the genus *Prumnopitys* Phil. for section *Stachycarpus* some years later.[10] Page[11] also agreed with Quinn[7] for the need to recognize 'fairly natural groupings which prove to have good geographic and probably evolutionary cohesion' and took the last few steps towards fulfilling Quinn's call for generic status for each section. He thus recognized *Podocarpus* section *Sundacarpus* as the (monotypic) genus

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Sundacarpus (Buchh. & Gray) C.N. Page, *Podocarpus* section *Polypodiopsis* as the genus *Retrophyllum* C.N. Page (a renaming of sect. *Decussocarpus* of de Laubenfels's illegitimate *Decussocarpus*), *Podocarpus* section *Nageia* as genus *Nageia* Gaertner and *Podocarpus* section *Afrocarpus* as genus *Afrocarpus* (Buchh. & Gray) C.N. Page.

While these changes strive to reflect natural (monophyletic) groupings of species, there are unavoidable nomenclatural complications that affect the users of botanical (and common) names. The raising of section *Afrocarpus* to the rank of genus affects one of South Africa's most prominent trees, the bastard or small-leaved yellowwood, *Podocarpus falcatus*, which (*sensu* Page[11]) is now named *Afrocarpus falcatus* (Thunb.) C.N. Page. When first proposed, South African botanists rejected this taxonomy as it was thought that the differences between *Afrocarpus* and *Podocarpus* were so insignificant that their separation was not justified,[12] a position that has remain unchanged.[13]

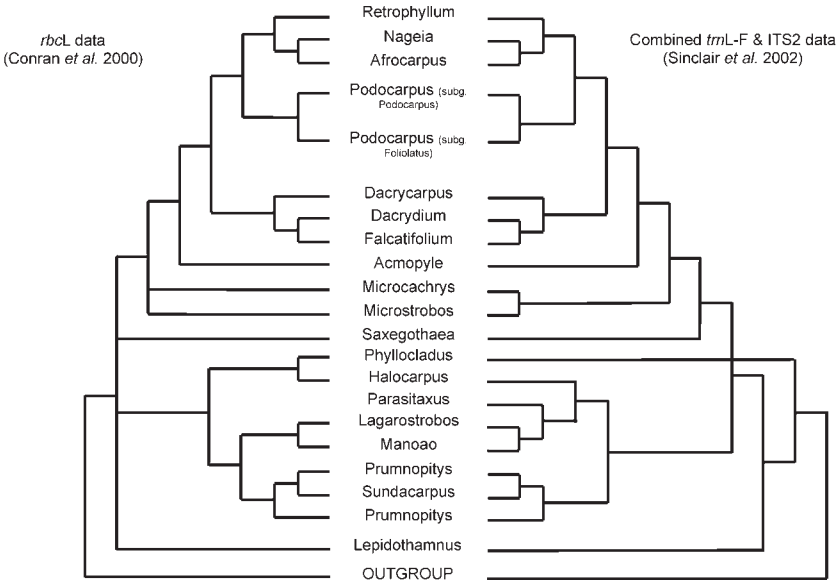

Fig. 1. A comparative summary of the genus-level phylogeny of genera of the Podocarpaceae, as obtained from the study based on *rbc*L sequence data (Conran *et al.*[15]) and combined *trn*L-F and ITS2 sequence data (Sinclair *et al.*[17]). Note that the only major discrepancy between the results of these two studies lies in the relationships of *Phyllocladus*, a result that might be an artefact arising from the use of different outgroups in the two studies.

Subsequent to these studies (and this rejection), several phylogenetic classifications of the Podocarpaceae have been undertaken, based on both morphological and molecular (DNA sequence) data. A cladistic analysis of morphological data[1] showed that *Podocarpus* section *Afrocarpus* was related to *Podocarpus* section *Nageia,* and that the remainder of *Podocarpus* (*Podocarpus s. str.*) was resolved as paraphyletic. However, it took only a single additional step in the cladistic analysis to render *Podocarpus* monophyletic, with a possible synapomorphy for a monophyletic *Podocarpus* then being the presence of overlapping bud scales. However, in a subsequent analysis based on nuclear 18S rDNA,[14] this relationship (i.e. *Afrocarpus* sister to *Nageia*) was once again retrieved, as was a paraphyletic *Podocarpus.* This latter result led Kelch[14] to consider the character of overlapping bud scales to be of dubious value.

A more recent study by Conran *et al.*[15] using data from the chloroplast gene *rbc*L included many more species than the studies of Kelch[1,14] and retrieved a considerably different set of relationships. The plastid *rbcL* data resolved a large clade of *Podocarpus* species (including the African *P. henkelii*). This clade was split into two subclades representing the subgenera *Podocarpus* and *Foliolatus* de Laub. that had been erected 15 years earlier on morphological grounds by de Laubenfels.[16] The genera *Afrocarpus, Nageia* and *Retrophyllum* were resolved as a sister clade to the main *Podocarpus* clade, and *Afrocarpus* was placed sister to *Nageia.*

The most recent study by Sinclair *et al.*,[17] based on both plastid *trn*L-F and nuclear Internal Transcribed Spacer2 (ITS2) sequence data, obtained a very similar result at the genus level. Although the species-level sampling is less than that of the *rbc*L study by Conran *et al.*,[15] there was some overlap in species sampled. These data retrieved the two subgenera of *Podocarpus*[16] as sister clades (forming a monophyletic *Podocarpus s. str.*) and *Afrocarpus, Nageia* and *Retrophyllum* once again form a separate sister clade to *Podocarpus s. str.* A summary cladogram of the relationships of the genera of the Podocarpaceae as evidenced by the studies of Conran *et al.*[15] and Sinclair *et al.*[17] is shown in Fig. 1.

It thus appears that there is considerable molecular (and morphological) evidence favouring the generic level recognition of *Afrocarpus* and the other genera as proposed by Page.[11] The small-leaved yellowwood should thus not be considered as a species of *Podocarpus* at all, and what was *Podocarpus falcatus* should now be called *Afrocarpus falcatus* (Thunb.) C.N. Page. The Podocarpaceae are now represented by two genera in Africa: *Podocarpus* and *Afrocarpus.*

Species delimitation in African Podocarpus

At the species level, another taxonomic issue relevant to southern Africa's *Podocarpus* species is that of the taxonomic status of *P. milanjianus.* Drummond[18] considered *P. milanjianus* to be the same species as *P. latifolius* and this treatment was subsequently adopted by Coates Palgrave[19] in his popular field guide (first and subsequent editions). In 1979, Hunt (from Kew Gardens, in correspondence with Gillett) noted that *P. milanjianus* should be considered a subspecies of *P. latifolius* (although it appears that this combination was never published). He based this on the fact that there was variation in leaf shape and size, male cones, peduncles and the length of their seeds, as well as geographical disjunctions. *P. latifolius* was considered as a southern African species, whereas *P. milanjianus* was considered to be restricted to eastern and central Africa.

Here we report on a small study to test this supposition, using DNA sequence data from the ITS2 region of nuclear DNA. As discussed above, this region has been used by Sinclair *et al.*[17] to determine generic-level relationships in species of Podocarpaceae, and, although it is rather short, it appears to be suitably variable at the species level.

Methods

Leaf samples of *P. latifolius* were collected from a range of localities, as indicated in Table 1. All except one sample is from South Africa. The specimen from Kenya is from a locality implying that it would be considered *P. milanjianus.* A DNA extract from a sample of *P. milanjianus* was obtained from the Royal Botanic Garden, Kew, and one of us (R.R.M.) provided material of *P. milanjianus* from the Royal Botanic Garden Edinburgh. In addition, one specimen each of *P. henkelii* (from a nursery source) and *A. falcatus* were also sequenced, and sequence data of *A. gracilior* were obtained from GenBank.

Genomic DNA was extracted using a CTAB method similar

Table 1. Voucher and locality details of specimens of *Podocarpus* sequenced in this study.

Taxon	Voucher	Locality
A. falcatus	E. Muller 21 (GRA)*	Alexandria forest, Eastern Cape, South Africa.
P. latifolius	N. Barker 1873 (GRA)	Knysna Forest, Prince Alfred's Pass, South Africa.
P. latifolius	N. Barker 1874 (GRA)	Knysna Forest, Montagu Pass, South Africa.
P. latifolius	N. Barker 1875 (GRA)	Nature's Valley Forest, South Africa.
P. latifolius	C. Peter s.n. (GRA)	Weza forest, KwaZulu-Natal, South Africa.
P. latifolius	T. Dold 4443 (GRA)	Beechamwood, Gatyana, Eastern Cape, South Africa
P. latifolius (=*P. milanjianus* according to distribution)	B. Bytebier 2328 (GRA; EAH)	Taita Hills, Kenya.
P. milanjianus	RBGE 19340272	Ex cult.; originally collected by G.N. Humphreys probably from Ruwenzori, Uganda (his no. 1446).
P. milanjianus	Chase 2482 (K)	Ex cult.; originally collected by J.D. Snowdon from Uganda.
P. henkelii	N. Barker 1909	Ex cult.; Sunnyside Nursery, Grahamstown, South Africa.

*EAH = East Africa Herbarium, Nairobi, Kenya; GRA = Selmar Schonland Herbarium, Grahamstown; K = Herbarium, Royal Botanic Gardens, Kew; RBGE = Royal Botanic Garden Edinburgh.

to that of Doyle and Doyle.[20] The ITS2 regions were initially amplified using the primers ITS3 (ref. 21) and GymR (ref. 22). Polymerase chain reactions (PCR) were undertaken at a range of magnesium concentrations from 1 mM to 5 mM. The PCR reaction mixes were made up of: 5 μl DNA template, 10 μl reaction buffer, 1 μM of each primer, 0.8 mM dNTPs, 1.5 units l *Taq* DNA polymerase (BIOLINE), 1–5 mM magnesium chloride and deionized water to make up volumes to 100 μl. The thermal cycling parameters were: 40 cycles of 45 s denaturation at 95°C, 45 s annealing at 55°C and 3 min extension at 72°C. Successful PCR amplifications were detected by electrophoresing 5 μl of the PCR product and 5 μl tracking dye in a 1% agarose gel, stained with ethidium bromide and visualized using a UV trans-illuminator.

The PCR products were purified using the QIAGEN QIA quick purification kit. An internal sequencing primer ('Podo-3') was designed that provided approximately 250 base pairs of sequence data (5'-TCA TCG AGT CTT TGA ACG CAA G-3'). Sequencing reactions were done using the ABI Big Dye Sequencing kit version 3.0, according to manufacturer's instructions. Samples were sequenced using an ABI 3100 genetic analyser. Sequences were checked using Sequencer version 3.01 (Gene Codes Corporation). The sequence data were then imported into DAPSA (DNA and protein sequence alignment) version 4.7 and aligned manually, along with sequence data from a second species of *Afrocarpus* (*A. gracilior*) from GenBank (AY083862) and an unpublished sequence of *P. milanjianus,* provided by one of us (R.R.M.). A Neighbor-Joining tree[23] based on absolute sequence differences was obtained from the aligned data using PAUP* v4.0b8.[24] This tree is shown in Fig. 2.

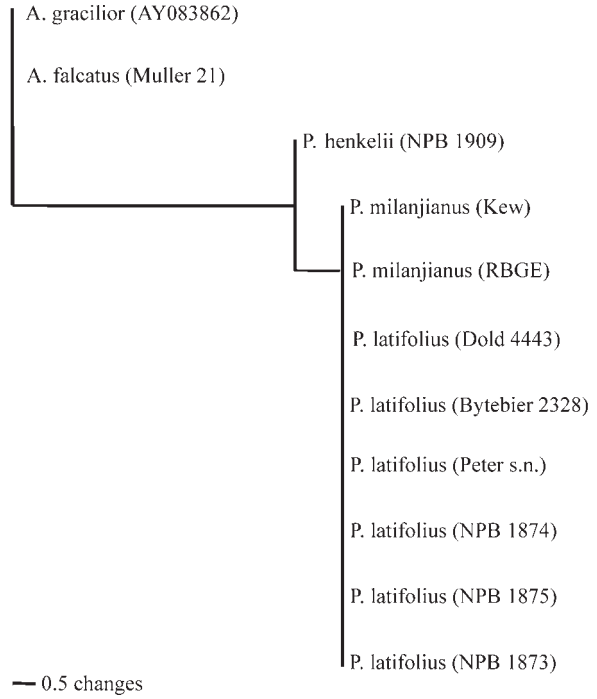

Fig. 2. A Neighbor-Joining phylogram (based on absolute sequence differences in 196 bases of ITS2 sequence data) of the five samples of *Podocarpus latifolius*, *P. milanjianus*, *P. henkelii* as well as representatives of *Afrocarpus*, *A. falcata* and *A. gracilior.* Vouchers or GenBank details as provided in Table 1 appear after the taxon names.

Results

The 5' and 3' ends of the alignment were trimmed to remove missing data from the analysis, and the resulting alignment data provided 196 base pairs of ITS2 sequence data. The ITS2 sequences of all the southern African specimens of *Podocarpus* were found to be identical. The sample from Kenya and the two samples from Uganda (Chase 2482 originally from Uganda and named at Kew as *P. milanjianus,* and the sample from the Royal Botanic Garden Edinburgh, also named as *P. milanjianus*) were also identical in ITS2 sequence to the southern African samples of *P. latifolius.* The single sample of *P. henkelii* differed from *P. latifolius* by a single base mutation (0.51%). Similarly, the two species of *Afrocarpus* (*A. falcatus* and *A. gracilior*) had identical ITS2 sequences, but these were markedly divergent from those of *P. latifolius* (eight base pair differences; 4.08%).

Discussion

At the generic level, the ITS2 data support the previous molecular studies in indicating the considerable 'genetic distance' between *Afrocarpus* and *Podocarpus,* providing further support for their recognition and acceptance as distinct genera. At the species level, ITS2 sequence data resolve *P. henkelii* as distinct from the *P. latifolius* samples but suggest that the recognition of *P. milanjianus* as distinct from *P. latifolius* is invalid. It thus appears that there is no genetic basis for the recognition of *P. milanjianus* as a distinct taxonomic entity on the basis of geographical partitioning, corroborating Drummond's[18] taxonomic considerations. This means that even though the samples of *P. latifolius* are from populations (forest patches) that are geographically isolated, gene flow is (or has been) possible. This can be explained by the hypothesis that the afromontane forests (of which *Podocarpus* species are a common component) were once far more widespread and continuous, and that the current patches are a result of recent fragmentation.[25]

While the timing of the fragmentation of Africa's afromontane forests is not known with certainty, comparatively recent cycles of Pleistocene aridification are thought to have resulted in the recent isolation of forest patches as refugia.[25,26] This scenario is congruent with our results, as it would imply that the current patches have not been isolated for a sufficiently long period of

time that would allow the accumulation of any substitutions within the (admittedly short) ITS2 region, and morphological variation is insufficient to be taxonomically informative with any degree of certainty.

An alternative explanation is that the species *P. latifolius* and *P. milanjianus* are valid but so similar that there is no ITS2 sequence variation between them. The latter hypothesis requires further consideration, since our data also show that two geographically widely separated species of *Afrocarpus* that can be separated morphologically (*A. falcatus* and *A. gracilior*) also have identical ITS2 sequences. Very similar results have been obtained in studies of Caribbean species of *Podocarpus* belonging to section *Pumilis* de Laub.; these also show genetic uniformity at the ITS2 sequence level, even though they can be distinguished on macro- and micromorphological characters.[27] Whichever explanation holds, the data presented here suggest that there is genetic uniformity (at the ITS2 sequence level) across Africa, as well as between species pairs of *Afrocarpus* and *Podocarpus,* and that alternative regions of DNA with greater variability need to be used. However, efforts in our labs at both Rhodes and Edinburgh to use the longer (and possibly more variable) ITS1 region in the Podocarpaceae have not proved successful owing to the amplification of multiple PCR products. DNA 'fingerprinting' approaches such as AFLPs or microsatellites may thus be required in order to study DNA-based variability at the species and population level in these taxa.

Over and above the *P. milanjianus–P. latifolius* question, there are certainly other taxonomic problems in African Podocarpaceae that need attention, including the delimitation of species pairs such as *Podocarpus henkelii–P. ensiculus* and a species complex that includes *Afrocarpus falcatus, A. gracilior, A. usambarensis, A. dawei,* as well as *A. mannii* from the island of São Tomé. It is obvious that DNA-based studies have contributed, and will continue to contribute significantly to our understanding of relationships in this intriguing family of gymnosperms.

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1. Kelch D.G. (1997). The phylogeny of the Podocarpaceae based on morphological evidence. *Syst. Bot.* **22**,113–131.
2. Farjon A. (2001). *World checklist and bibliography of conifers,* 2nd edn. Royal Botanic Gardens, Kew, Surrey.
3. Brummitt R.K., Mill R.R. and Farjon A. (2004). The significance of 'it' in the nomenclature of three Tasmanian conifers: *Microcachrys tetragona* and *Microstrobos niphophilus* (*Podocarpaceae*), and *Diselma archeri* (Cupressaceae). *Taxon* **53**, 529–539.
4. Leistner O.A. (1966). Podocarpaceae. *Flora of Southern Africa* **1**, 34–41.
5. Buchholz J.T. and Gray N.E. (1948). A taxonomic revision of *Podocarpus* I. The sections of the genus and their subdivisions with special reference to leaf anatomy. *J. Arn. Arb.* **29**, 49–63.
6. Mill R.R. and Weston P. (2004) (1613–1614). Proposals to reject the names *Polypodiopsis* and *Polypodiopsis muelleri* (*Plantae vasculares, incertae sedis*). *Taxon* **53**, 203–205.
7. Quinn C.J. (1970). Generic boundaries in the Podocarpaceae. *Proc. Linn. Soc. NSW* **94**, 166–172.
8. de Laubenfels D.J. (1969). A revision of the Malesian and Pacific rainforest conifers, I. Podocarpaceae, in part. *J. Arn. Arb.* **50**, 274–369.
9. de Laubenfels D.J. (1972). Gymnospermes. In *Flore de la Nouvelle-Calédonie et Dépendances,* 4, eds A. Aubreville and J-F. Leroy. Muséum National d'Histoire Naturelle, Paris.
10. de Laubenfels D.J. (1978). The genus *Prumnopitys* (Podocarpaceae) in Malesia. *Blumea* **24**, 189–190.
11. Page C.N. (1989). New and maintained genera in the conifer families Podocarpaceae and Pinaceae. *Notes R. Bot. Gard. Edinb.* **45**, 377–395.
12. Leistner O.A., Smith G.F. and Glen H.F. (1995). Notes on *Podocarpus* in southern Africa and Madagascar. *Bothalia* **25**, 233–245.
13. Glen H.F. (2000). Podocarpaceae. In *Seed Plants of Southern Africa: families and genera,* ed. O.A. Leistner. *Strelitzia* **10**, 30–31.
14. Kelch D.G. (1998). Phylogeny of Podocarpaceae: comparison of evidence from morphology and 18S rDNA. *Am. J. Bot.* **85**, 986–996.
15. Conran J.G., Woods G.M., Martin P.G., Dowd J.M., Quinn C.J., Gadek P.A. and Price R.A. (2000). Generic relationships within and between the gymnosperm families Podocarpaceae and Phyllocladaceae based on an analysis of the chloroplast gene *rbc*L. *Aust. J. Bot.* **48**, 715–724.
16. de Laubenfels D.J. (1985) A taxonomic revision of the genus *Podocarpus. Blumea* **30**, 251–278.
17. Sinclair W.T., Mill R.R., Gardner M.F., Woltz P., Jaffré T., Preston J., Hollingsworth M.L., Ponge A. and Möller M. (2002). Evolutionary relationships of the New Caledonian heterotrophic conifer, *Parasitaxus usta* (Podocarpaceae), inferred from chloroplast *trn*L-F intron/spacer and nuclear rDNA ITS2 sequences. *Pl. Syst. Evol.* **233**, 79–104.
18. Drummond R.B. (1975). A list of trees, shrubs and woody climbers indigenous or naturalised in Rhodesia. *Kirkia* **10**, 229–285.
19. Coates Palgrave K. (1977). *Trees of Southern Africa,* 1st edn. Struik, Cape Town.
20. Doyle J.J. and Doyle J.L. (1987). A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochem. Bull.* **19**, 11–15.
21. White T.J., Bruns T., Lee S. and Taylor J. (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In *PCR Protocols: a guide to methods and applications,* eds M.A. Innis, D.H. Gelfand, J.J. Sninsky and T.J. White, pp. 315–322. Academic Press, San Diego, CA.
22. Liston A., Robinson W.A., Oliphant J.M. and Alvarez-Buylla E.R. (1996). Length variation in the nuclear ribosomal DNA internal transcribed spacer region of non-flowering seed plants. *Syst. Bot.* **21**, 109–120.
23. Saitou N. and Nei, M. (1987). The Neighbor-Joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* **6**, 514–525.
24. Swofford D.L. (2000). PAUP: Phylogenetic Analysis Using Parsimony. Version 4.0b3a. Smithsonian Institution, Washington, D.C.
25. Linder H.P. (1998). Numerical analyses of African plant distribution patterns. In *Chorology, Taxonomy and Ecology of the Floras of Africa and Madagascar,* eds C.R. Huxley, J.M. Lock and D.F. Cutler, pp. 67–86. Royal Botanic Gardens, Kew, Surrey.
26. Griswold C.E. (1991). Cladistic biogeography of afromontane spiders. *Aust. Syst. Bot.* **4**, 73–89.
27. Stark Schilling D.M. (2004). *Taxonomic studies of Caribbean and Central American species of* Podocarpus *subgenus* Podocarpus. M.Sc. thesis, Royal Botanic Garden Edinburgh/University of Edinburgh.