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PHYLOGEOGRAPHY OF *SCHOTIA* (FABACEAE): RECENT EVOLUTIONARY PROCESSES IN AN ANCIENT THICKET BIOME LINEAGE

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Schotia has a southern African distribution with a strong affinity to the thicket biome and is a potential indicator of thicket biogeography. This study uses DNA sequence data (from the chloroplast and nuclear genomes) to infer interspecific phylogenetic relationships, to elucidate the evolutionary history of *Schotia*, and to extrapolate findings to the history of the thicket biome. Four species of *Schotia* represented by multiple samples were nonmonophyletic for both chloroplast and nuclear markers. These results may be due to hybridization and/or incomplete lineage sorting, which also suggests a recent origin of *Schotia* morphospecies. The center of genetic and taxonomic diversity of *Schotia* is the Eastern Cape and partly the Western Cape, coinciding with the hub of present-day thicket distribution. This region is hypothesized as being a refugial area for *Schotia* (and the associated thicket biome) during glacial periods, with expansion beyond this region during interglacial periods.

Keywords: cpDNA, Fabaceae, ITS, Leguminosae, phylogeography, *Schotia*, southern Africa, thicket biome.

Online enhancement: appendix.

Introduction

In a recent study of global Fabaceae (Leguminosae) biogeography, Schrire et al. (2005) suggested that many persistent legume elements are endemic to a broadly defined "succulent biome," a vegetation type that comprises succulent-rich, dry tropical forest and thicket that is depauperate in grasses. These authors argue for a circum-Tethyan origin for many persistent legume groups in this vegetation type during the early Tertiary (Schrire et al. 2005; Bruneau et al. 2008). According to Bruneau et al. (2008), the early Eocene age of the Detarieae along with some deeply diverging continental disjunctions are suggestive of vicariance events.

In southern Africa, thicket vegetation corresponds to the succulent biome of Schrire et al. (2005). This is a dense formation of evergreen and weakly deciduous shrubs and low trees (2–5 m) that are often spiny and covered with vines. It is centered in the southeastern part of southern Africa and is the dominant formation in the central and eastern Little Karoo and the major river valley systems between the Great Escarpment and the Indian Ocean coast. Thicket also forms mosaics with vegetation from surrounding biomes and is widespread throughout the subcontinent (Vlok et al. 2003; Cowling et al. 2005). In many respects, southern African thicket appears to be transitional in nature, with multiple affinities. Early studies on southern African vegetation included thicket vegetation within fynbos, succulent karoo, forest, and savanna biomes (Acocks 1953; Moll and White 1978; Boucher and Moll 1980; Huntley 1984; Rutherford and Westfall 1986; Midgley et al. 1997). This led to confusion regarding thicket affinities and evolutionary relationships with adjacent vegetation (Cowling 1984; Lubke 1996; Vlok et al. 2003; Cowling et al. 2005; Hoare et al. 2006).

The thicket biome has a moderately rich diversity of plants from a wide range of taxonomic groups. Vlok et al. (2003) focused on a subtype of thicket known as subtropical thicket and found ~1600 plant species associated with this vegetation, of which 20% were endemic. Thicket endemics appear to not be random assemblages (both taxonomically and biologically), and they are strongly associated with a limited number of succulent families (e.g., Aizoaceae, Asphodelaceae, Crassulaceae, Euphorbiaceae, and Apocynaceae). In contrast, many thicket shrub and tree species have relatively wide distributions that extend in satellite populations in thicket vegetation beyond the boundaries of the thicket biome (Cowling et al. 2005; fig. 1). These satellite populations suggest that the thicket biome is ancient and has experienced substantial shifts in distribution ranges.

The thicket vegetation of southern Africa is therefore hypothesized to be of an ancestral vegetation type that prevailed throughout the subtropical, semiarid parts of the world during the Eocene, at ~40 Ma, when major thicket lineages originated (Cowling et al. 2005). Thicket is thus considered to be the "mother vegetation" of many plant lineages that have diversified in the modern southern African biomes (Cowling and Pierce 2009).

However, there is no fossil evidence to support this, and molecular dating studies at the family level (Wikström et al.

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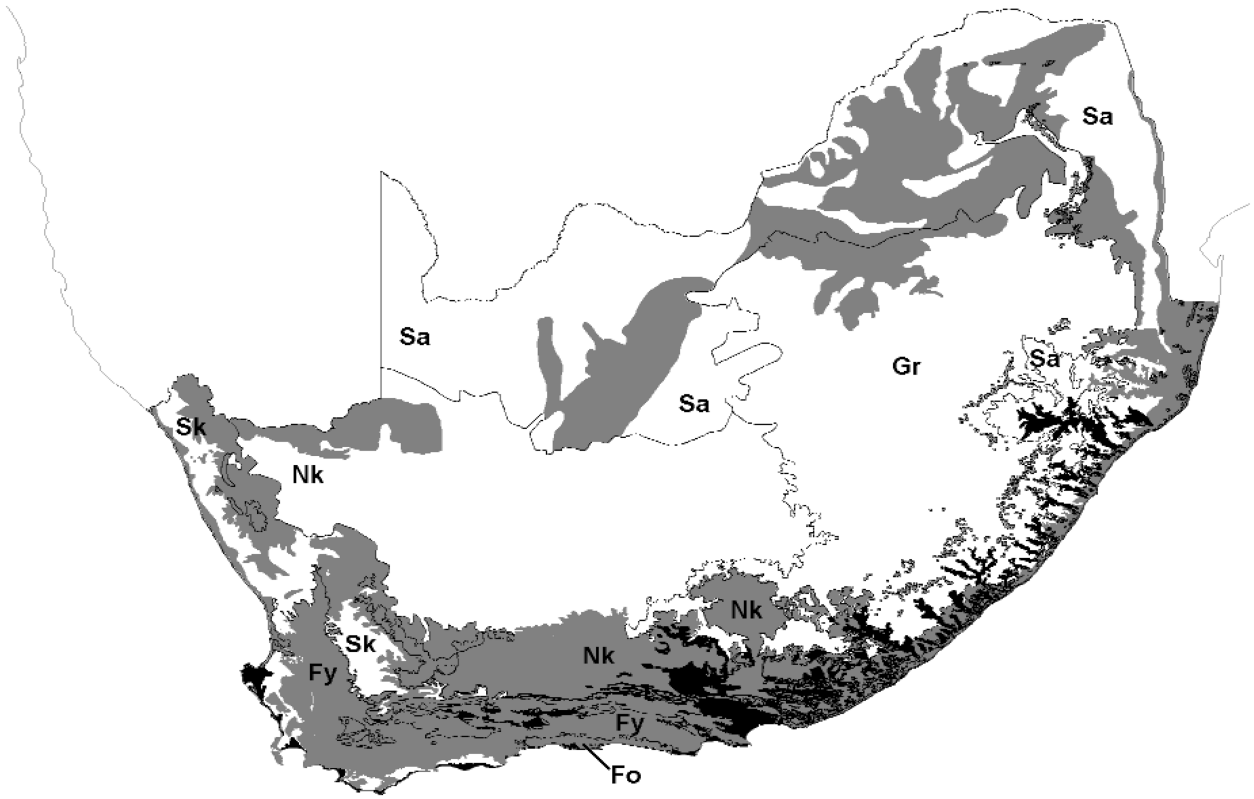

Fig. 1 Map showing distribution of the thicket biome (black areas) and biomes that contain thicket vegetation (i.e., mosaics; gray areas) in southern Africa (Lesotho, Swaziland, and South Africa). *Fo* = forest, *Fy* = fynbos, *Gr* = grassland, *Nk* = Nama-Karoo, *Sa* = savanna, *Sk* = succulent karoo (from Cowling et al. 2005).

2001, 2003; Davies et al. 2004; Bell et al. 2005; Magallón and Castillo 2009) are of limited use due to the exclusion of endemic thicket families or lineages. Schrire et al. (2005) found that southern African thicket is home to two persistent legume genera: *Schotia* Jacq. and *Umtiza* T.R. Sim. Cowling et al. (2005) and Schrire et al. (2005) suggest that *Schotia* is one of the early thicket lineages.

Here we report on a phylogenetic and phylogeographic study of *Schotia*, using both chloroplast (cp) and nuclear ribosomal (nr) internal transcribed spacer (ITS) DNA sequence data. The aims of this study are to elucidate species-level phylogenetic relationships and to use interspecific genetic diversity to assess historical range expansions, contractions, and isolation of the thicket biome in South Africa.

The Genus Schotia

Schotia (family Fabaceae, subfamily Caesalpiniodeae, tribe Detarieae) is a small genus of trees that is confined to southern Africa, ranging from southern Zimbabwe and Mozambique to southern South Africa and Namibia. Within southern Africa, it is predominantly associated with the thicket biome, but with range extensions into other regions and biomes where it is invariably associated with thicket or forest-thicket mosaics. The most recent taxonomic revision of *Schotia* by Ross (1977) recognizes four species: *Schotia afra* (L.) Thunb. (with two varieties, *S. afra* var. *afra* and *S. afra* var. *angustifolia* [E. Mey.] Harv.), *Schotia latifolia* Jacq., *Schotia brachypetala* Sond., and *Schotia capitata* Bolle. All four species are associated with the thicket biome or mosaics of thicket vegetation found within other southern African biomes. Ross (1977) also noted the existence of a possible new species and recorded what he considered to be hybrids between *S. latifolia* and *S. afra*. In addition, he reported considerable variation within species that makes them difficult to separate in the absence of flowering material (Ross 1977). *Schotia afra* is a particularly widespread species, and it shows a disjunction between its main center of distribution in the Eastern Cape and southern regions of Namibia and the Northern Cape of South Africa, where populations associated with the Orange River and its surrounds (the Gariep Centre of endemism) have been found (Jürgens 1997; van Wyk and Smith 2001; Curtis and Mannheimer 2005). This disjunction suggests either a far more expansive thicket biome distribution in the past or a considerably long-distance, animal-facilitated dispersal. This species produces large pods that are consumed and dispersed by large herbivores (e.g., elephants; Sigwela 2005).

Material and Methods

Taxon Sampling Strategy

Sampling focused on the thicket regions and mosaics of southern Africa. Multiple samples were obtained for all spe-

cies except *Schotia capitata*, for which there were only two samples. We attempted to cover as much of the distribution range of these species as possible. Most samples collected have an accompanying herbarium voucher that is deposited in the Selmar Schonland Herbarium (Rhodes University, Grahamstown). However, some DNA samples were used that had no herbarium voucher and no collector number, and as such they are tagged as *sine numero* (sn). Voucher specimen data and GenBank accession numbers used in this study are provided in the appendix in the online edition of the *International Journal of Plant Sciences*. The identification of several of these samples proved difficult. Accurate identification requires flowering material, but in several instances we had only fruiting or leaf material at our disposal. Consequently, there is some uncertainty associated with some of our identifications (especially those distinguishing between *Schotia latifolia* and *Schotia afra* and putative hybrids). In such cases, samples were tentatively referred to as *Schotia* sp., along with their respective species affinity or their hybrid status. It must also be noted that, given the nature of our results, the use of species names is of little value anyway. We also refrained from identifying the samples of *S. afra* to variety level, as a phenetic study had indicated the characters used to distinguish these taxa to be of dubious value (results not shown).

DNA Extraction, Amplification, and Sequencing

DNA was extracted from leaf material dried in silica gel (Chase and Hills 1991) or, occasionally, from fresh material, using a modified hot hexadecyl trimethyl-ammonium bromide (CTAB) method of Doyle and Doyle (1987). Two noncoding chloroplast DNA (cpDNA) regions were used, the *trnL-trnF* spacer and the *psbA-trnH* intergenic spacer (hereafter referred to as the *trnL-F* and *psbA-trnH* spacers, respectively). The *trnL-F* spacer is one of the most widely used cpDNA markers in phylogenetic reconstruction (Shaw et al. 2005) and has been widely used at the species level in legumes (e.g., Haston et al. 2005; Kenicer et al. 2005; Torke and Schaal 2008). It has also proven useful at the intraspecific level for some legume taxa (e.g., Sotuyo et al. 2007). The *psbA-trnH* spacer is usually short, but it can be useful to discriminate between closely related species (Chandler et al. 2001; Seigler et al. 2006; Carine et al. 2007) and at the intraspecific level in Fabaceae (e.g., Sotuyo et al. 2007). In addition, the nr ITS region was used to provide an independent source of sequence data. This region is usually more variable than the chloroplast region, and it has been used extensively in legume systematics (Aïnouche and Bayer 1999; Allan and Porter 2000; Barker et al. 2000; Lavin et al. 2001; Hughes et al. 2002; Percy and Cronk 2002; Murphy et al. 2003; Schnabel et al. 2003; Schrire et al. 2003; Kenicer et al. 2005; Fougére-Danezan et al. 2007; Javadi et al. 2007; Ribeiro et al. 2007; Torke and Schaal 2008). It has also been used at the infraspecific level in legume studies (Cubas et al. 2006).

The *trnL-F* spacer was amplified and sequenced using primers E and F (Taberlet et al. 1991). The *psbA-trnH* spacer was amplified and sequenced using primers psbA and trnH (Sang et al. 1997). The ITS region (ITS1, 5.8S, and ITS2) was amplified using the flanking primers ITS18 and ITS26 (Käss and Wink 1997). The ITS18 primer was modified as described by Beyra-Matos and Lavin (1999). These were used as flanking primers for amplification. Internal primers (both forward and reverse) were used for sequencing. These were primers ITS1 and ITS4 (White et al. 1990), as well as primers Chrys5.8F and Chromo5.8R (Barker et al. 2005; McKenzie et al. 2006). Polymerase chain reaction (PCR) amplicons were purified using Promega Wizard or Invetec kits according to the manufacturers' instructions. Cleaned products were sequenced using the BigDye Terminator sequencing kit, version 3.1 (Applied Biosystems, Foster City, CA). In all instances, sequencing was performed in both forward and reverse directions. The cycle-sequencing products were precipitated using a sodium acetate/EDTA protocol. Sequencing was performed on an ABI 3100 genetic analyzer (Applied Biosystems) at Rhodes University's Sequencing Unit.

Outgroup Selection

Fougére-Danezan et al. (2007) found that *Barnebydendron riedelii* (Tul.) J.H. Kirkbr. is most probably sister to *Schotia* (also see Mackinder 2005). We did not have *Barnebydendron* material at our disposal, nor were the sequences available on databases for all the markers used in this study. Thus, in the combined cpDNA analyses, *Aphanocalyx pectinatus* (A. Chev.) Wieringa was used as the outgroup. *Aphanocalyx* is a close African relative of *Schotia*, with sequence data for all markers. ITS sequences for *Barnebydendron* were available from GenBank. Additional outgroups included *Goniorrhachis marginata* Taub. and *Brandzeia filicifolia* Baill. (see appendix).

Sequence Editing and Alignment

For each accession, contiguous sequences were assembled, checked, and edited using Sequencher, version 4.2.2. (Gene Codes Corporation). All sequences were deposited in GenBank (appendix). Alignment of sequences was straightforward, and sequences were aligned manually in MacClade, version 4.06 (Maddison and Maddison 2000). Gaps were inserted intuitively on the basis of visual inspection of the sequences to minimize the number of nucleotide differences among sequences. Following alignment, the data sets for the two cpDNA markers were combined; thus, two data sets were analyzed (a combined cpDNA and an ITS data set).

Phylogenetic Analyses

Distance, maximum parsimony (MP), and Bayesian inference (BI) methods were used in phylogenetic reconstruction. The neighbor-joining (NJ) method (Saitou and Nei 1987) was used to construct distance trees. The model of nucleotide substitution for the NJ analysis was generated using Modeltest 3.7 (Posada and Crandall 1998) and incorporated into reconstructions performed using PAUP*, version 4.0b10 (Swofford 2002). Nodal support was evaluated by generating 10,000 NJ bootstrap replicates.

MP searches were performed with PAUP* 4.0b10 (Swofford 2002) in conjunction with PAUPRat, version 1 (Sikes and Lewis 2001). The parsimony-ratchet procedure of Nixon (1999) was implemented. Each ratchet analysis consisted of

20 independent runs of 200 iterations. The trees found in the separate searches were pooled ($n = 4000$) and served as starting trees for a heuristic searches in PAUP* 4.0b10 employing tree bisection-reconnection (TBR) branch swapping. All trees were used to construct a strict consensus tree. Parsimony bootstrap analyses for each data partition consisted of 1000 heuristic bootstrap replicates with MAXTREES set at 4000. In all analyses this proved to be computationally prohibitive, and so MAXTREES was reduced to 500.

Before performing BI analyses, data sets were partitioned. This was easily achieved for the cpDNA data, as both markers were amplified independently. The ITS partitions were determined using the ITS sequence of *S. afra* (GenBank accession AY955774). Models of sequence evolution for each partition was determined using the Akaike Information Criterion (AIC) as implemented in the program MrModeltest, version 2.2. (Nylander 2004). BI was performed using MrBayes, version 3.1.1 (Huelsenbeck and Ronquist 2001). This analysis was conducted with four Monte Carlo Markov Chains (three heated and one cold) that were run for 5 million generations and sampled every 100th generation. The output files were examined to determine when log likelihood values stabilized. These burn-in generations were excluded when constructing the BI trees. Posterior probabilities (PP) were estimated by constructing a 50% majority rule consensus tree in PAUP* 4.0b10. Trees were visualized using TreeView, version 1.6.6 (Page 1996).

Congruence of the cpDNA and ITS data sets was assessed using the partition homogeneity (ILD) test (Farris et al. 1994). The ILD test was implemented in PAUP* 4.0b10 (using 1000 replicates, MAXTREES set at 100, and TBR branch swapping). Additionally, conflict was assessed by visually examining nodal support of the main lineages. Clades that were not well supported (bootstrap values $< 70\%$, posterior probabilities < 0.95) were considered to be nonconflicting and warranted merger of data sets (e.g., Mason-Gamer and Kellogg 1996).

Phylogenetic Networks

Neighbor-net (NN) is a fast, consistent, and relatively efficient distance-based network construction method that allows for graphical representation of conflicting phylogenetic signals and interpretation of evolutionary histories that are not treelike (Bryant and Moulton 2004; Bryant et al. 2007). This method is not always readily informative, but it does produce a splits graph that potentially indicates the part(s) responsible for the complexity. These parts can be further scrutinized with more specialized analyses (Bryant and Moulton 2004). NN splits graphs have been used with varying success to detect reticulate history and hybridization (McBreen and Lockhart 2006; Carine et al. 2007; Frajman and Oxelman 2007; Killian et al. 2007; Whithead and Lockhart 2007; De Lange et al. 2008; Grimm and Denk 2008; Weiss-Schneeweiss et al. 2008). Splits graphs were constructed using the NN algorithm with uncorrected p-distances in SplitsTree, version 4.10 (Huson 1998; Huson and Bryant 2006). The plastid markers are inherited as a single unit and were analyzed together. Both the cpDNA and the ITS matrices were modified before analysis. Outgroups were excluded. Thereafter, the matrices were rechecked and redundant gaps (mainly outgroup-linked indels) were removed.

Results

cpDNA Phylogeny

A summary of sequence characteristics of the cpDNA data sets (individual and combined) is presented in table 1. There is a low rate of plastid evolution in Detarieae, which has resulted in poorly resolved relationships at the generic level (Bruneau et al. 2008). An ILD test showed that the cpDNA data partitions were not significantly incongruent ($P = 0.257$). This is explained in part by the *trnL-F* spacer only very weakly resolving any relationships, and it validated the merger of cpDNA markers. Analysis of cpDNA data with the MP method produced 4000 trees of 101 steps (consistency index [CI] = 0.812, retention index [RI] = 0.877).

The BI tree was the best resolved and was chosen to depict topological results (fig. 2). Five cpDNA lineages were recovered (clades A–E; fig. 2). Lineages with bootstrap (BS) values $\geq 70\%$ and posterior probability (PP) values ≥ 0.95 were considered to be well supported. *Schotia* as a lineage is not well supported for the cpDNA data set. It is also immediately ob-

Table 1

Sequence Characteristics of the *trnL-F* and *psbA-trnH* Spacers and the Internal Transcribed Spacer (ITS) Region

Parameter	*trnL-F*	*psbA-trnH*	Combined cpDNA (*trnL-F* + *psbA-trnH*)	ITS
Ingroup/s + *Schotia*:				
No. samples	74	63	51	71
No. bp in alignment	580	452	1028	748
No. variable characters (%)	59 (10.2)	46 (10.2)	81 (7.9)	252 (33.9)
No. parsimony-informative characters (%)	12 (2.1)	25 (5.5)	30 (2.9)	90 (12.0)
Schotia:				
No. samples	70	62	50	67
No. bp in alignment	546	409	954	630
No. variable characters (%)	18 (3.3)	34 (8.3)	50 (5.2)	80 (12.7)
No. parsimony-informative characters (%)	6 (1.1)	24 (5.9)	28 (2.9)	47 (7.5)

Note. cpDNA = chloroplast DNA; bp = base pairs.

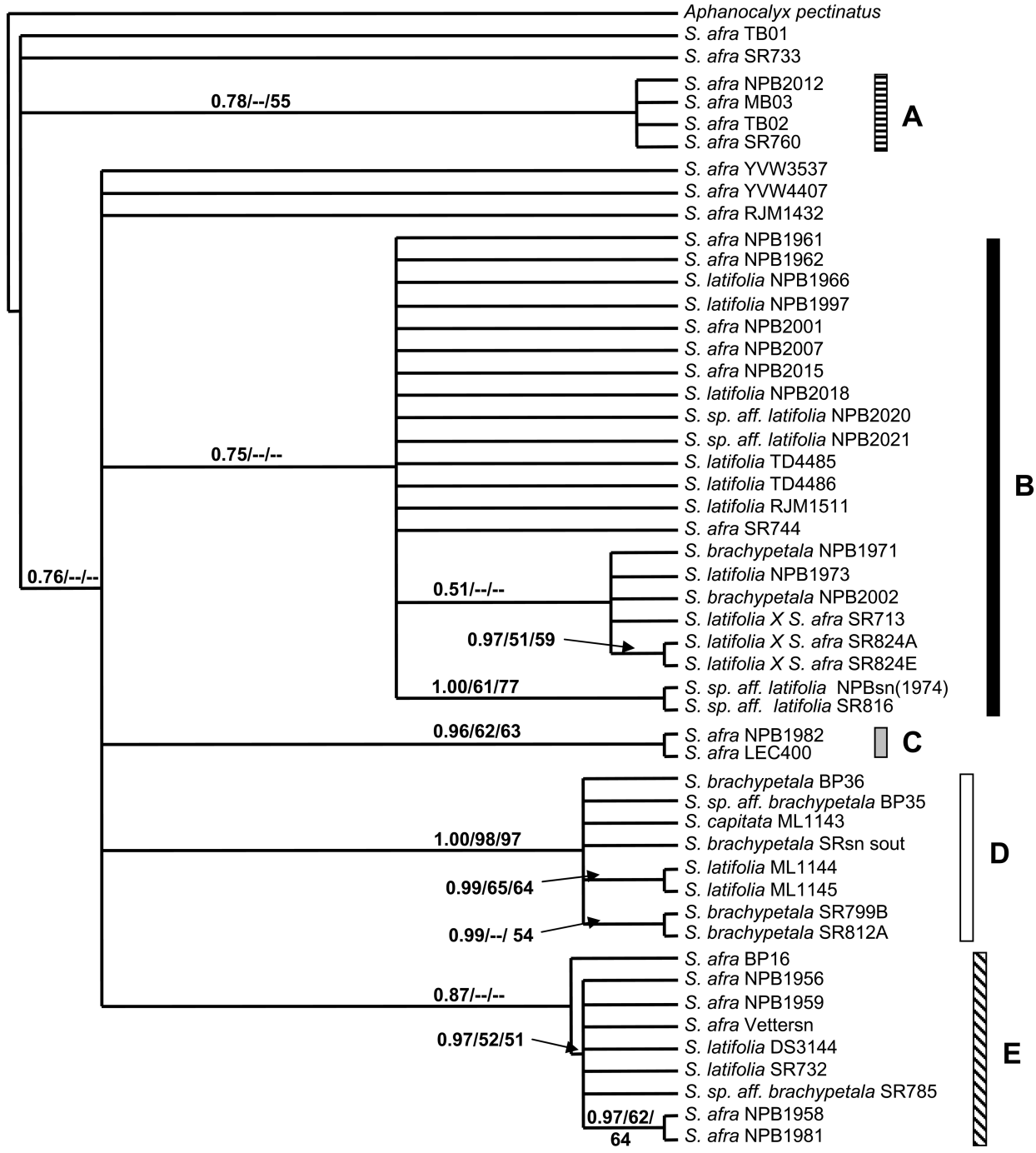

Fig. 2 Bayesian inference (BI) tree of the combined chloroplast DNA (cpDNA) data sets estimated using the following models: F81 (*trnL-F*) and GTR+I (*psbA-trnH*; determined by the Akaike Information Criterion). Major groups are denoted by bars on the right (labeled *A–E*; discussed in text). Numbers above the branches or next to arrows indicate posterior probability (BI)/bootstrap (maximum parsimony)/bootstrap (neighbor-joining) values.

vious that none of the four species in the genus are retrieved as monophyletic. Two samples of *Schotia afra* (TB01 and SR733) are placed at the base of the topology and are not in any of the delimited clades. Clade A is composed only of *S. afra* ($n = 4$). It was not retrieved in the MP analysis, and it was weakly supported in the BI and NJ analyses (fig. 2). The two unplaced taxa (TB01 and SR733) and clade A are collectively sister to all the other samples, which are in a large clade with weak BI analysis support and no support for MP and NJ analyses (fig. 2). Four clades and three unplaced samples of *S. afra* (YVW3517, YVW4407, and RJM1432) comprise this large clade. Of the four clades, clade B, which is the largest clade ($n = 22$), is composed of a mix of *S. afra*, *Schotia brachypetala*, *Schotia latifolia*, *Schotia sp. aff. latifo-*

lia, and *S. latifolia* × *S. afra*. This clade is not retrieved in the MP and NJ analyses, and it is weakly supported in the BI analysis (fig. 2). Clade C, the smallest lineage, is composed of two *S. afra* samples and is weakly supported in the MP and NJ analyses but has strong support in the BI analysis (PP = 0.96; fig. 2). Clade D ($n = 8$) is well supported in all analyses (BI: PP = 1.00; MP: BS = 98%; NJ: BS = 97%) and is composed of *S. brachypetala*, *Schotia capitata*, *S. latifolia*, and *Schotia sp. aff. brachypetala* (fig. 2). Clade E ($n = 9$) is composed of *S. afra*, *S. latifolia*, and *S. sp. aff. brachypetala* and receives no support in the MP and NJ analyses. It is only weakly supported in the BI analysis (fig. 2).

ITS Phylogeny

PCR products generated for the ITS region were resolved as single bands with no size differences. Additionally, no double peaks or ambiguous base calls were found in electrophenograms of ITS sequences. Because of lack of evidence of paralogs and the relatively large amount of samples included in this study, attempts at cloning were not pursued and all sequences are considered to be functional copies and not pseudogenes (e.g., Razafimandimbison et al. 2004).

A summary of the ITS sequence characteristics is given in table 1. In Detarieae, nuclear markers (including ribosomal loci) are more rapidly evolving than plastid markers (Bruneau et al. 2008). Variability and parsimony-informative characters for ITS are much higher than the combined cpDNA markers, irrespective of whether the outgroups are excluded (table 1). The MP analysis produced 4000 trees of 391 steps (CI = 0.795, RI = 0.864). As with the cpDNA analyses, the BI tree is chosen to depict ITS results.

Schotia was retrieved as a single lineage with no support in the ITS phylogeny (fig. 3). Five lineages were recovered for the ITS region (labeled clades 1–5; fig. 3). Of these, two are resolved as sister lineages (clades 1 and 2), with this lineage in a polytomy along with the remaining three clades. Clade 1 ($n = 11$) is composed of *S. afra*, *S. brachypetala*, *S. capitata*, *S. latifolia*, and *S. sp. aff. brachypetala*. This clade is not well supported in all of the analyses (fig. 3). Clade 2 is the largest lineage, with 31 samples. It is composed mostly of *S. afra* and *S. latifolia* × *S. afra*. It is well supported in the BI (PP = 1.00) and MP (BS = 96%) analyses but poorly supported in the NJ analysis (fig. 3). Clade 3 ($n = 6$) is composed of *S. latifolia*, *S. sp. aff. latifolia*, and *S. sp. aff. brachypetala* and is well supported in all of the analyses (BI: PP = 0.98; MP: BS = 78%; NJ: BS = 90%; fig. 3). Clade 4 ($n = 15$) is composed of *S. afra*, *S. brachypetala*, *S. latifolia*, *S. sp. aff. brachypetala*, and *S. latifolia* × *S. afra*. This clade is moderately supported in the BI analysis (PP = 0.90), weakly supported in the NJ analysis, and not supported in the MP analysis (fig. 3). Clade 5 is the smallest lineage, with four samples, and it is composed of *S. latifolia*, *S. sp. aff. afra*, and *S. sp. aff. latifolia*. It is well supported in all of the analyses (BI: PP = 0.98; MP: BS = 83%; NJ: BS = 88%; fig. 3).

Visual examination revealed that cpDNA clades C and D and ITS clades 2, 3, and 5 were well supported (either by bootstrap and/or posterior probability values). The cpDNA and ITS phylogenies showed a high degree of topological incongruence, despite some cpDNA clades (A, B, and E) and ITS clades (1 and 4) having poor or no support. Only one lineage in each phylogeny is broadly congruent, that is, clade D (cpDNA) and clade 1 (ITS). Interestingly, clade D is well supported in the cpDNA phylogeny but clade 1 is not supported in the ITS phylogeny. Additionally, an ILD test indicated that the data partitions were significantly incongruent ($P = 0.001$). On the basis of these findings, the cpDNA and ITS data sets were kept separate.

Phylogenetic Networks

Attempts were made to relate the splits-graph lineages (and clusters) to the clades recovered in the phylogenies. The cpDNA splits graph revealed two main lineages (fig. 4). One of the main lineages is labeled D (fig. 4), while the other main lineage is composed of several clusters (labeled A–C and E; fig. 4). Lineage D corresponds to clade D of the cpDNA phylogeny. Lineages A, C, and E also broadly correspond to the cpDNA clades (fig. 2). The splits-graph lineage B mostly corresponds to clade B of the cpDNA phylogeny, except for a single taxon (sample 43 = SR744, *S. afra*) that is nested within lineages A and C (arrowed in fig. 4). Lastly, the unplaced taxa of the cpDNA phylogeny are concentrated in close proximity to lineage A, with a single unplaced taxon (sample 21 = YVW4407, *S. afra*) placed between lineages C and E (black arrows in fig. 4). All of these splits-graph lineages (except lineage C) have an internal network structure.

Two main lineages were delimited for the ITS splits graph (fig. 5). One of the main lineages is labeled 2 (fig. 5), while the other main lineage is composed of several clusters (labeled 1, 3, 4, and 5; fig. 5). Lineage 2 corresponds to clade 2 of the ITS phylogeny. Lineages 1, 3, 4, and 5 correspond to ITS phylogeny clades 1, 3, 4, and 5, respectively. All ITS splits-graph lineages revealed a high degree of internal network complexity, which is indicative of reticulation. As several *Schotia* species have a known history of hybridization, the reticulate nature of the splits graphs could possibly be reflecting this. Surprisingly, none of the cpDNA lineages are similar to the ITS lineages except for splits-graph cpDNA lineage D and ITS lineage 1, a pattern also recovered in the cpDNA and ITS phylogenies. This makes hybridization impossible to disentangle. By constructing and analyzing matching data sets, we attempted to eliminate the effects of dissimilar sample sets that may have confounded results. The patterns we recovered were broadly similar to those in the full data sets (not shown), and like the full data sets, they could not be used to disentangle hybridization. The possibility of other phenomena (such as recombination within a population) that can lead to evolutionary histories that are nontreelike with reticulate patterns (Bryant et al. 2007) cannot be ruled out.

Discussion

Species Nonmonophyly

Fougére-Danezan et al. (2007) found that *Schotia latifolia* was sister to *Schotia afra* and *Schotia brachypetala*. However, Fougére-Danezan et al. (2010) found that *S. afra* was

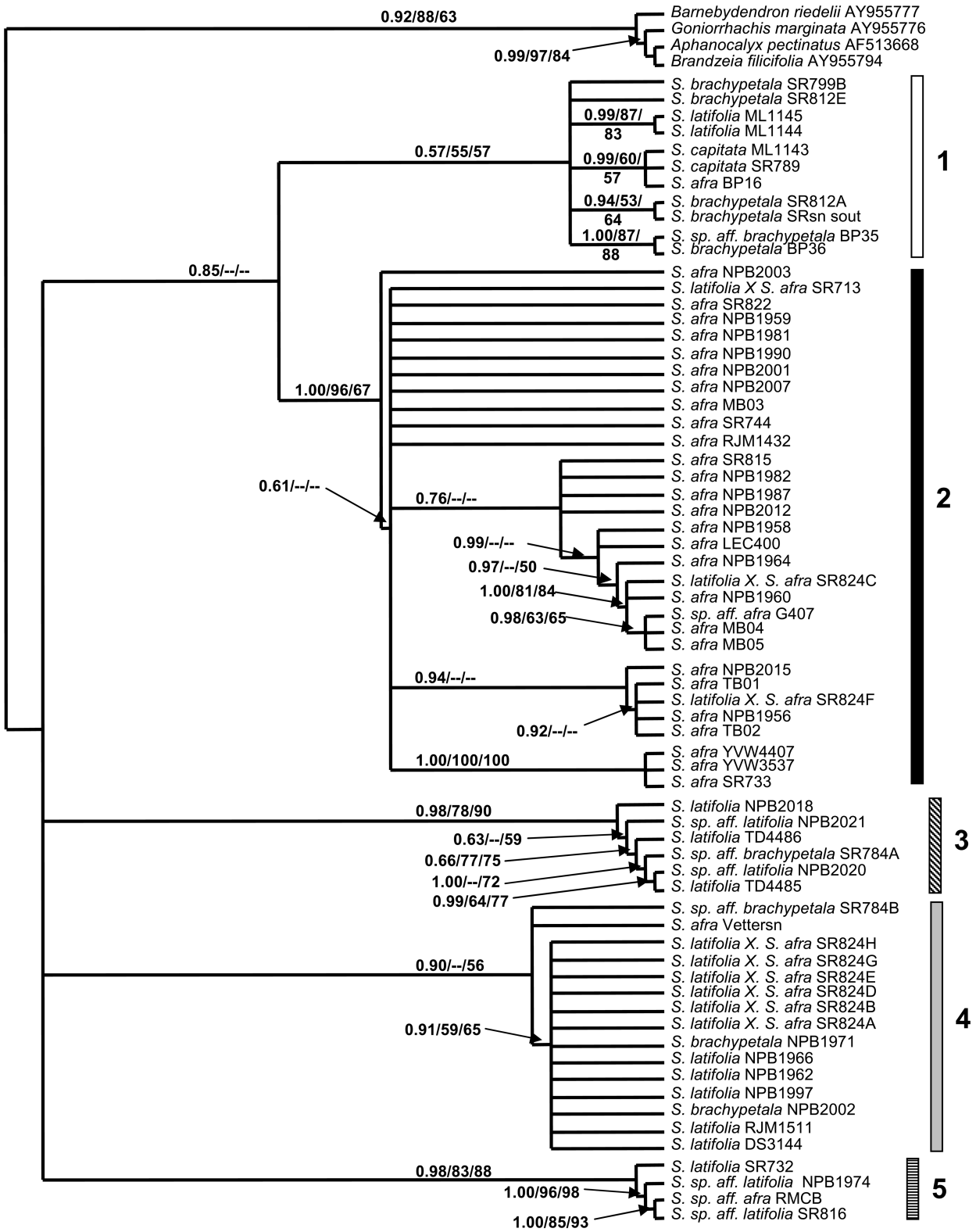

Fig. 3 Bayesian inference (BI) tree of the internal transcribed spacer (ITS) region estimated using the following models: GTR+G (ITS1), SMY (5.8S), and HKY+G (ITS2; determined by the Akaike Information Criterion). Groups denoted by bars on the right (labeled *1–5*) are discussed in the text. Numbers above the branches or next to arrows indicate posterior probability (BI)/bootstrap (maximum parsimony)/bootstrap (neighbor-joining) values.

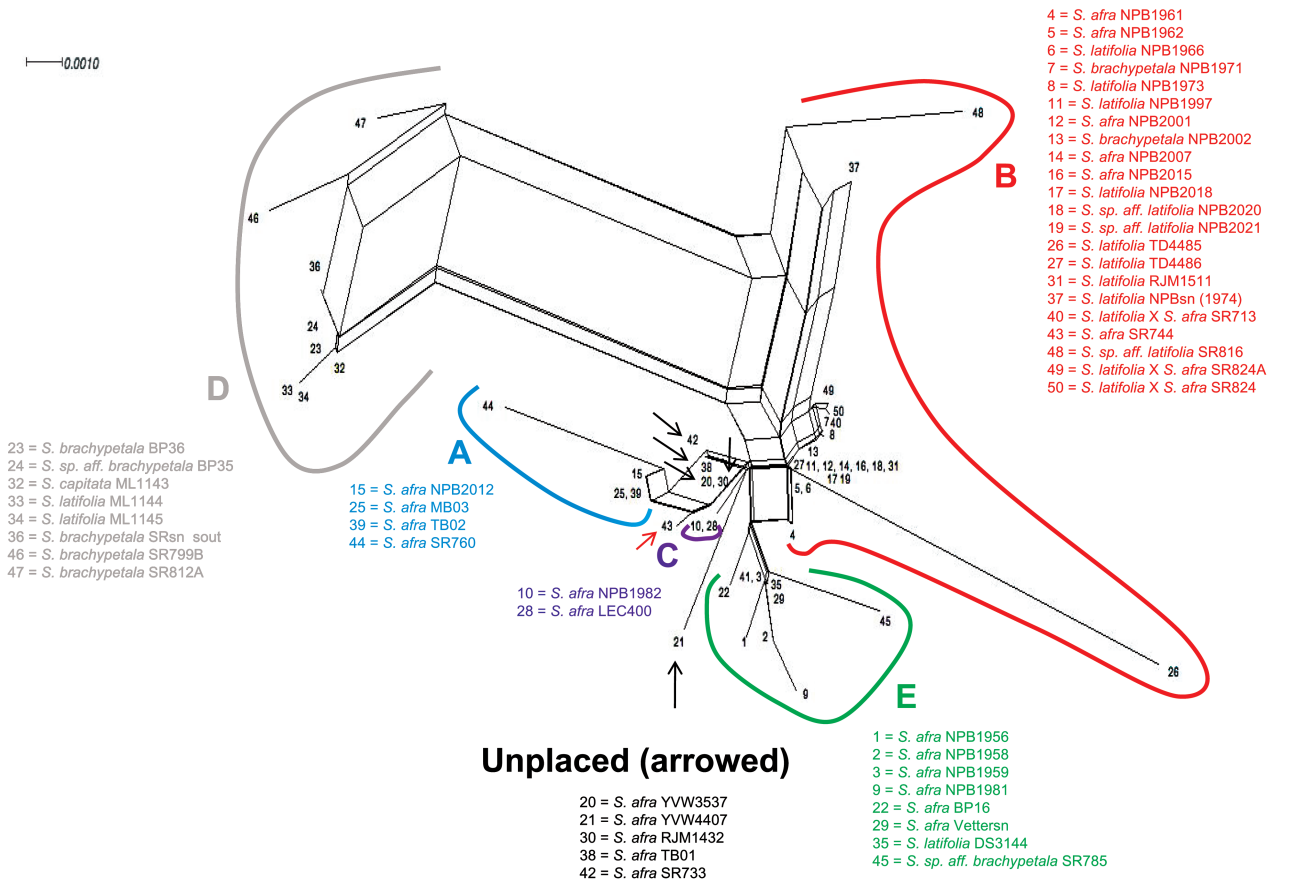

Fig. 4 Splits graph for chloroplast DNA (cpDNA) sequences of *Schotia*. Two major branches were recovered (i.e., lineage D and other lineages [A, B, C, and E]). The major lineages recovered are more or less congruent with the major cpDNA clades (see fig. 2). Lineage A = blue, lineage B = red, lineage C = purple, lineage D = gray, and lineage E = green; unplaced taxa are indicated with black arrows.

sister to *S. latifolia* and *S. brachypetala*. This study, which included all species in the genus as well as much denser sampling for three of the four *Schotia* species, revealed that multiple samples were resolved as nonmonophyletic for both markers. Even in strongly supported lineages (clades C and D in the cpDNA analysis and clades 2, 3, and 5 in the ITS analysis), there was species nonmonophyly. Two explanations may account for nonmonophyly: either the alpha taxonomy is poorly resolved and/or the molecular markers used here are not reflecting the history of the morphological entities.

Schotia is a small, taxonomically well-studied genus. However, exact species boundaries are confusing in many instances (Ross 1977). Despite this, *S. afra* is easy to identify, even in the vegetative state. This study has found that the taxon is clearly not monophyletic and appears in several of the clades in both cpDNA and ITS analyses. The incongruence between the evolutionary history inferred from the molecular data and the currently recognized morphospecies may be attributable to hybridization and/or incomplete lineage sorting. Hybridization and/or incomplete lineage sorting may not be mutually exclusive, and the two are difficult to disentangle (Comes and Abbott 2001; Goldman et al. 2004; Church and Taylor 2005; Ramdhani et al. 2009).

Hybridization

Codd (1956) and Ross (1977) recorded putative *Schotia* hybrids in areas of sympatry. Additionally, Ross (1977) recorded considerable variation within species. Molecular-based phylogenetics studies are increasingly showing that hybridization is an important phenomenon in angiosperm evolution (Vriesendorp and Bakker 2005; Barber et al. 2007; Watanabe et al. 2008; Ramdhani et al. 2009). Nucleotide ITS additivity is an indicator of different paternal and maternal copies originating from hybridization events (e.g., Carine et al. 2007). We examined a hybrid population from Seaview (Port Elizabeth; SR824) in greater detail to check for ITS additivity. Six ITS sequences were generated for individuals in this population (SR824A, -B, -D, -E, -G, and -H, all placed in clade 4 [fig. 3]). In this hybrid population, we found no evidence for ITS additivity.

The potential to interbreed (Codd 1956; Ross 1977), the extensive morphological variation (Ross 1977), the discordant nuclear and chloroplast phylogenies, and the reticulate network patterns all appear to indicate that hybridization may be (or has been) a strong evolutionary factor in *Schotia*. Hybridization and reticulation can result in complex patterns

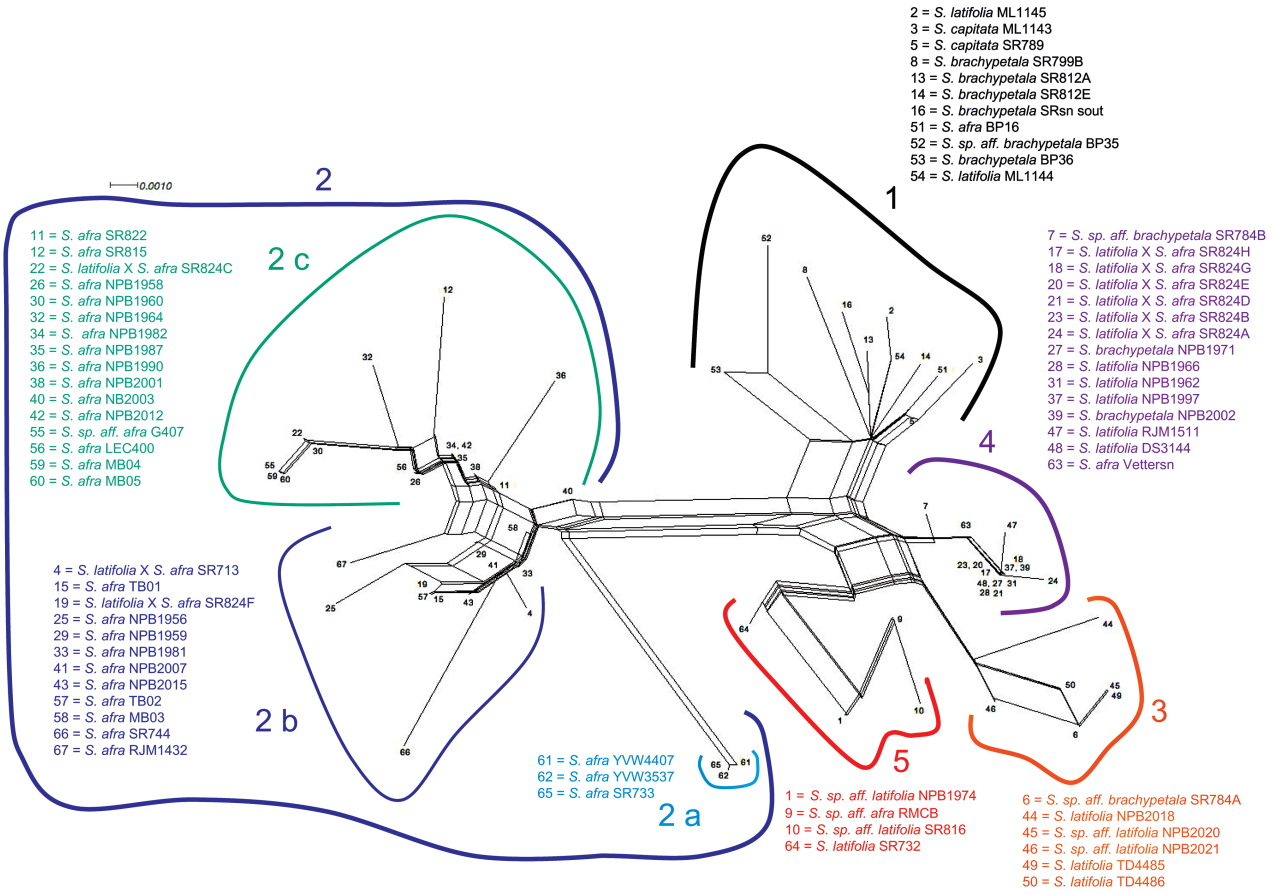

Fig. 5 Splits graph for internal transcribed spacer (ITS) sequences of *Schotia*. Two major branches were recovered (i.e., lineage 2 and other lineages [1, 3, 4, and 5]). The major lineages recovered are congruent with the major ITS clades (see fig. 3). Lineage 1 = black, lineage 2 (*2a–2c*) = blue, lineage 3 = orange, lineage 4 = purple, and lineage 5 = red.

that may lead to morphological plasticity (Mummenhoff et al. 2004; Watanabe et al. 2008). *Schotia* morphospecies appear to not have fully developed reproductive barriers, thereby allowing hybridization and back-crossing (i.e., mixing of haplotypes and ribotypes) to occur.

Incomplete Lineage Sorting

Incomplete lineage sorting from a polymorphic ancestral gene pool can result in a morphospecies being nonmonophyletic. Determining the role of lineage-sorting processes requires species ages. This will show if haplotype divergence pre- or postdates speciation. If divergence predates speciation, then lineage sorting could account for haplotype sharing from a polymorphic ancestor. However, if speciation predates haplotype divergence, interspecific hybridization and introgression must be considered (McKinnon et al. 2001).

Fabaceae is considered to be a tropical family with a late Cretaceous origin (59–79 Ma; Wojciechowski et al. 2004; Schrire et al. 2005; Bruneau et al. 2008; Bello et al. 2009). Bruneau et al. (2008) estimated the age of major caesalpinioid clades to be at 34.0–56.5 Ma, with the stem node fixed at 65 Ma. Detarieae, which is an early branch in the legume phylogeny, is dated at 53.6 Ma (crown-node estimate; Bruneau et al. 2008), with *Schotia* and *Barnebydendron* among the most basal genera (Fougére-Danezan et al. 2007, 2010). We attempted dating the ITS phylogeny, using BEAST, version 1.4.8 (Drummond et al. 2002, 2006; Drummond and Rambaut 2007). A mean rate for a woody perennial was used on the basis of the ITS rates of Kay et al. (2006). Our results (not shown) indicated a *Barnebydendron-Schotia* split at ~12–13 Ma, which is considered to be a gross underestimation of age. Consequently, we decided to use the result of Bruneau et al. (2008), which provides a broad picture of nodal ages for Detarieae with extensive sampling and robust dating. Close examination of the dated phylogeny of Bruneau et al. (2008) reveals that, although *Schotia* is an old lineage (stem age, ~48–49 Ma), the crown age for *Schotia* is ~5–6 Ma. Despite these age estimates, we cannot determine whether haplotype divergence pre- or postdated speciation, but we suspect recent speciation (see below).

This raises a very intriguing question: how do we explain the long stem age (~48–49 Ma) and the recent crown age (~5–6 Ma)? We link the long stem with increasing Neogene aridification in southern Africa (Cowling et al. 2005; Cowl-

ing and Pierce 2009). The long stem suggests high levels of extinction in divergent *Schotia* lineages with increasing Neogene aridification (hence the overall persistence of a large stem age for *Schotia*). The young crown age appears to be linked to Pleistocene glacial cycles, and it reflects the most recent divergent branch within *Schotia* (see below).

Consequently, we consider speciation to have occurred relatively recently, with haplotype divergence predating speciation, so incomplete lineage sorting accounts for haplotype sharing from a polymorphic ancestral lineage with a large stem age. However, in *Schotia*, present in situ hybridization is also observed. If morphospecies are relatively young, we cannot rule out the possibility that reproduction barriers have insufficient time to reinforce species boundaries allowing for hybridization. Thus, we conclude that both incomplete lineage sorting and hybridization are responsible for the extensive haplotype sharing that is expressed as species nonmonophyly in the cpDNA and ITS phylogenies.

Geographical Distribution of Phylogenetic Lineages

Mapping the distribution of the five cpDNA and ITS lineages revealed several interesting (and, because of the phylogenetic incongruence, conflicting) geographical patterns. Patterns from the cpDNA analysis (fig. 6) show that clade A has an Eastern Cape province–Albany region distribution. Clade B has a wide distribution that is centered in the Albany region of the Eastern Cape and extends north into the Pondoland region and southwest into the Karoo. Clade C has an inland Albany region distribution (Eastern Cape). Clade D has a wide distribution in northeastern South Africa. It extends from northern KwaZulu-Natal to the Soutpansberg (Limpopo Province). Clade E has a very wide distribution, from northern KwaZulu-Natal (Pongola) to the Eastern Cape, with a disjunction in the Northern Cape province. The disjunction of Clade E (i.e., Eastern Cape and Northern Cape disjunct pattern) matches the disjunction of *S. afra* (and numerous other taxa) described by Jürgens (1997).

The distributions of the five ITS lineages are shown in figure 7. Clade 1 is similar in distribution to cpDNA clade D (the only similar lineage for the cpDNA and ITS phylogenetic analyses), which extends from northern KwaZulu-Natal to the Soutpansberg (Limpopo Province). Clade 2 has a wide distribution, from the Albany region of the Eastern Cape and extending southwest into the Karoo and coastal regions of the Western Cape Province. Clade 3 has a coastal Pondoland region distribution that extends into the Albany region (Eastern Cape). Clade 4 extends from southern KwaZulu-Natal into the Pondoland and Albany regions, with a disjunction in the Northern Cape Province. Clade 5 appears to have a restricted Albany region distribution (Eastern Cape). Each of these regions may have been occupied by refugial patches of frost-sensitive subtropical thicket during glacial periods.

Biogeographical Hypothesis

Thicket is presumed to be the ancestral vegetation type that covered most of southern Africa until ~12–15 Ma.

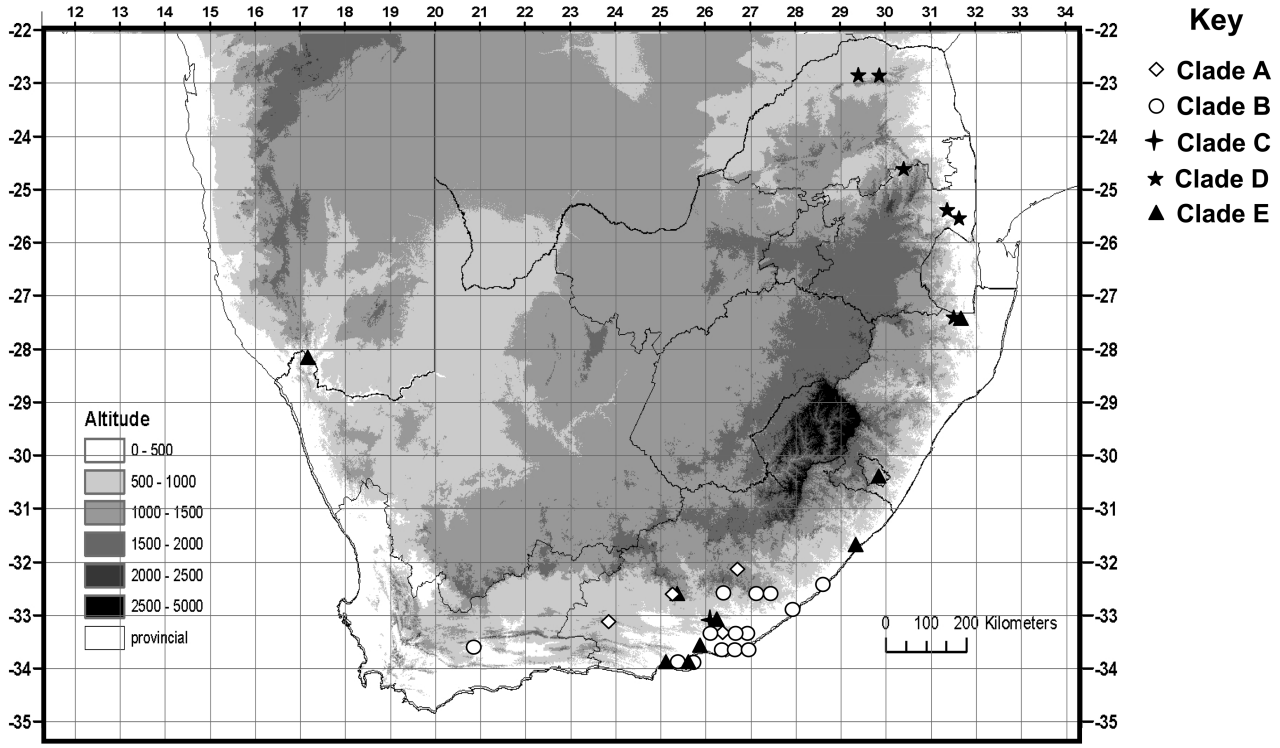

Fig. 6 Map showing distribution of major chloroplast DNA clades (*A–E*) in southern Africa. Map shows Lesotho, Swaziland, and provinces of South Africa.

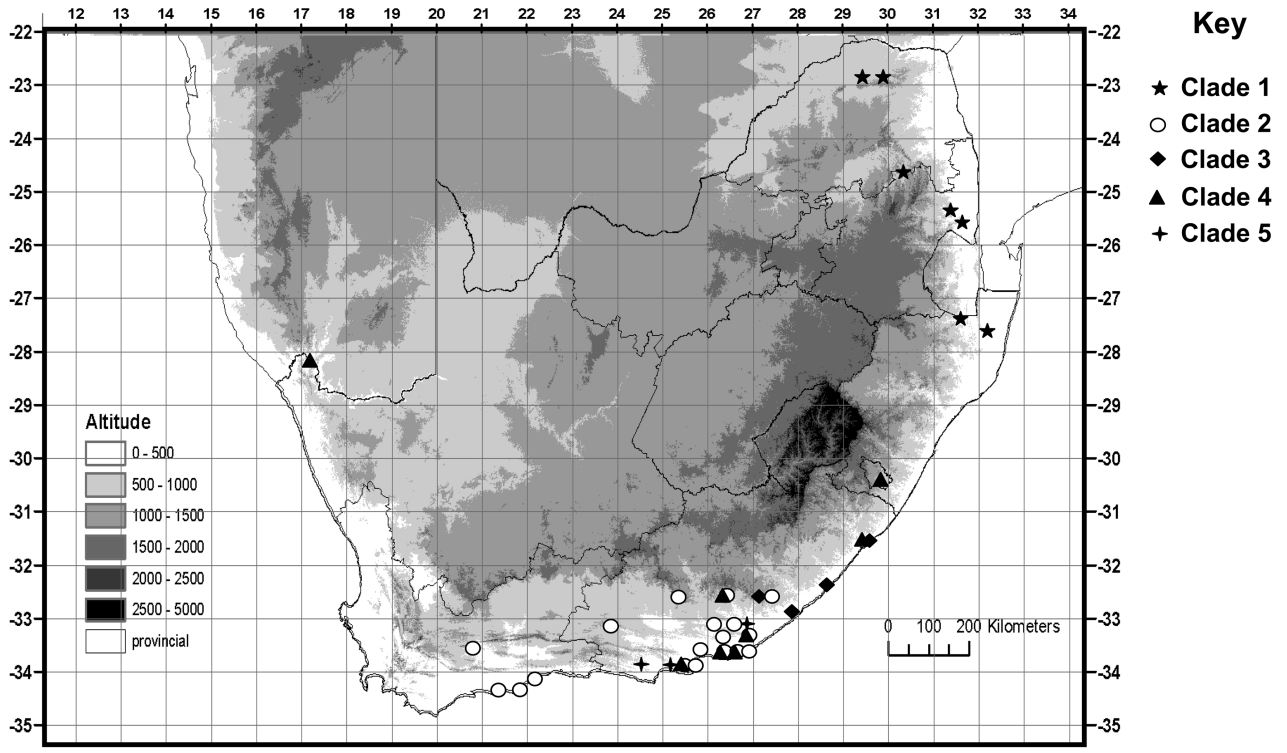

Fig. 7 Map showing distribution of major internal transcribed spacer clades (*1–5*) in southern Africa. Map shows Lesotho, Swaziland, and provinces of South Africa.

Thicket and forest started to retreat with increasing Neogene aridification in southern Africa (Cowling et al. 2005; Cowling and Pierce 2009) as fynbos, karooid, grassland, and savanna vegetation types evolved during alternating dry and wet conditions (~5.3–12 Ma). Since the Pleistocene (1.8 Ma), cyclic ice ages have endured for 100,000-year intervals, punctuated by brief, 10,000-year-long warmer periods (Cowling and Pierce 2009). Southern Africa experienced limited glaciation during these ice ages (Lewis 2008), but the impact of these cycles on vegetation and flora was considerable (Goldblatt 1978), and the present-day thicket distribution is presumed to be shaped by paleoclimatic cycles (Cowling et al. 2005).

Since *Schotia* is a thicket associate, it is reasonable to hypothesize that the ancestral area for *Schotia* was geographically restricted when thicket was retreating with increasing Neogene aridification in southern Africa (Cowling et al. 2005; Cowling and Pierce 2009). The large *Schotia* stem age supports this hypothesis. Additionally, the younger *Schotia* crown age appears to reflect recent diversification driven by Pleistocene glacial cycles. During the last glacial maximum (LGM) 18,000 years ago, conditions were cool and dry and vegetation belts were lowered in altitude by ~1000 m, responding to a ~5°C drop in temperature (Scott 1983; van Zinderen Bakker 1983; Vogel 1983; Scott et al. 1997; Lewis 2008). The sea level on the eastern coast of South Africa dropped by a maximum of ~120 m below the present level during the LGM (Ramsay and Cooper 2002). This altitudinal shift would have had important consequences for vegetation zones and thicket. Climatic deterioration would have resulted in the contraction and fragmentation of thicket into refugial areas.

During wetter and warmer interglacial periods (similar to the present-day climate), thicket expanded in range distribution and interdigitated with the other surrounding vegetation types (Cowling et al. 2005; Cowling and Pierce 2009). Figure 8*A* shows the present distribution of the thicket biome (black), biomes that contain thicket mosiacs (gray), and the distribution of *Schotia* in southern Africa (blue). Evidence for a postglacial (altithermal) expansion can be seen in the form of the wide coverage of cpDNA clade E (green area in fig. 8*B*) and ITS clade 4 (pink area in fig. 8C), which are now disjunct distributions. These clades could have extended from the Richtersveld in the northwest corner of South Africa (the sample *S. afra*, Vetter sn) to Pongola in KwaZulu-Natal and possibly farther north, suggesting a once-widespread, continuous tract of thicket vegetation in southern Africa. Subsequent to this expansion, thicket (and *Schotia*) became centered in the Eastern Cape and partly in the Western Cape. This is presently the hub of the thicket biome, which coincides with the center of diversity for *Schotia* species and genetic diversity (both cpDNA and ITS; solid green outline in fig. 8*D*). Four of five cpDNA haplotypes and ITS ribotypes are centered in this region. Also shown are the hypothesized postglacial migration/expansion routes northward along the extremities of the subcontinent (fig. 8*D*). In both the cpDNA and the ITS phylogenies, clade D and clade 1, respectively, were broadly congruent (in sample composition); this is reflected in the mapping of these clades (purple areas in fig. 8*B*

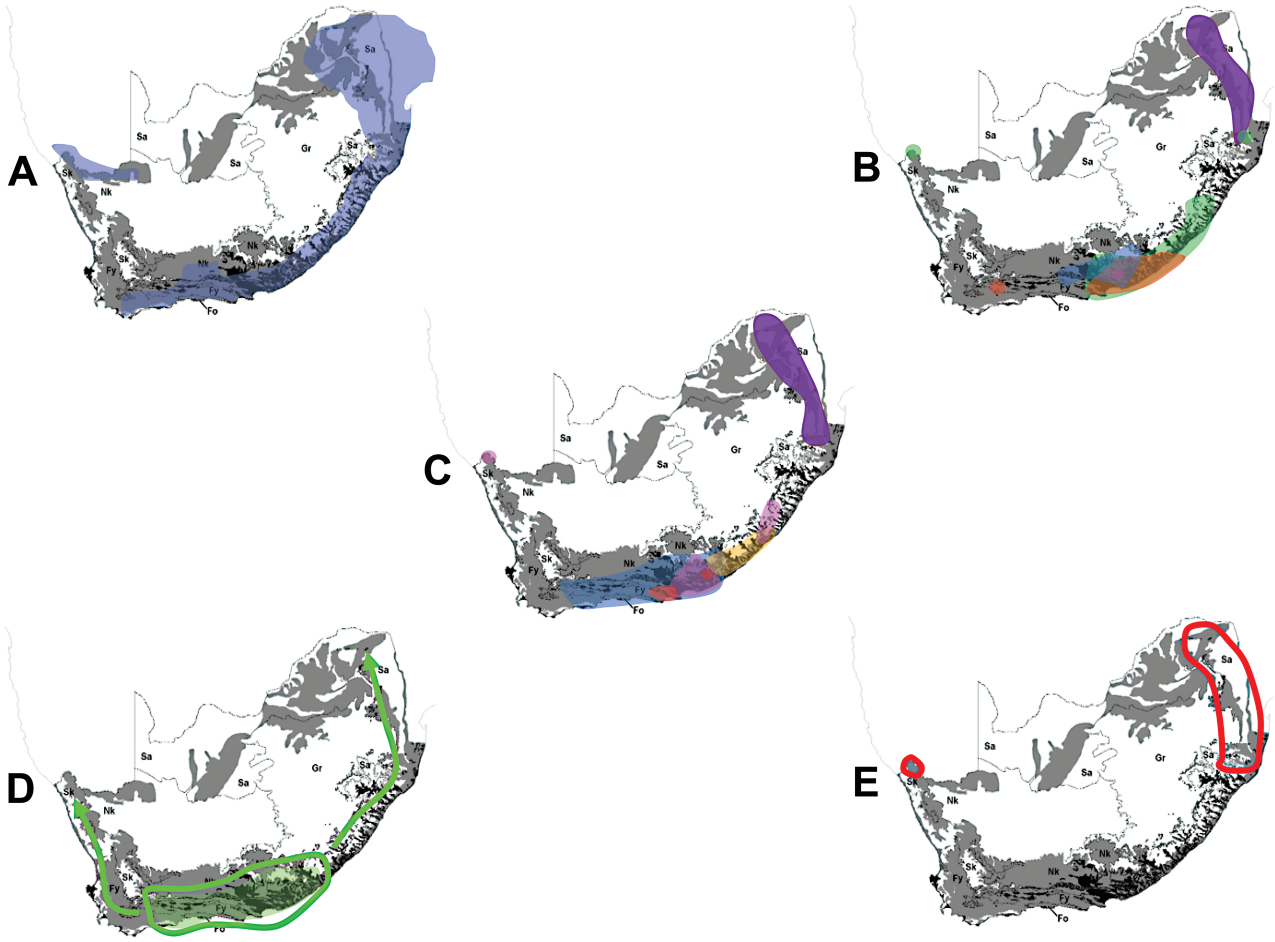

Fig. 8 Maps showing the hypothesized biogeographical interpretation for *Schotia* in southern Africa. *A*, Distribution of the thicket biome (black), biomes that contain thicket mosaics (gray), and the distribution of *Schotia* in southern Africa (blue). *B*, Distribution of the major chloroplast DNA (cpDNA) clades recovered (clade A = blue, clade B = red, clade C = pink, clade D = purple, clade E = green; see fig. 6 for greater detail). *C*, Distribution of the major internal transcribed spacer (ITS) clades recovered (clade 1 = purple, clade 2 = blue, clade 3 = orange, clade 4 = pink, clade 5 = red; see fig. 7 for greater detail). *D*, Map showing the present distribution hub of thicket (solid green outline) that coincides with the center of diversity for *Schotia* (green shading). Also shown are the hypothesized postglacial migration/expansion routes northward along the extremities of South Africa. *E*, Map showing subsequent isolation of *Schotia* in the northeastern and northwestern extremes of southern Africa.

and 8*C*). We suspect that *Schotia* populations are experiencing genetic isolation in the northeastern parts of South Africa (fig. 8*E*).

Other thicket associates (e.g., *Nymania capensis* [Thunb.] Lindb., *Carrisa haematocarpa* [Eckl.] A. DC., and section *Glaucophyllum* of *Pelagonium*) that also have a distinct disjunct distribution in the lower part of the Orange River basin (Jürgens 1997) require phylogeographic examination to determine whether any common pattern (thus invoking a common cause for this disjunction) can be determined. However, in light of our findings of nonmonophyletic species, incongruence between cpDNA and nrDNA, the geographic distribution patterns of the clades retrieved, and the possible role of incomplete lineage sorting and hybridization, we propose that extant species of *Schotia* are of recent origin and that their evolutionary history is linked to Pleistocene climatic cycles and vegetation changes. However, our results are too poorly resolved to make a precise identification of areas that have been thicket refugia.

Several reasons may explain why, despite the use of multiple markers, we did not recover a more refined genetic signature to address the main aim of this study. Southern Africa did not experience severe temperature extremes during recent ice ages (Lewis 2008). We suggest that the lack of severe glaciations and temperature extremes are responsible for refugia that are not totally isolated in a nonglaciated landscape. This and the fact that southern Africa was geomorphologically stable during the Pleistocene (1.8 Ma; Partridge and Maud 1987) would provide some "buffering" and stability during the glacial cycles, accommodating an ecological setting that does not allow for complete isolation of populations. This effectively means that well-defined phylogeographical patterns are more challenging to recover in southern Africa (e.g., Barker et al. 2005; Bergh et al. 2007) compared with the

Northern Hemisphere, where the split between glacial and interglacial phases of climatic oscillations is more extreme and better characterized (e.g., Hewitt 2004; Waltari et al. 2007; Bennett and Provan 2008; Hofreiter 2008), allowing for easier inference of phylogeographical structure and patterns (e.g., Aoki et al. 2006; Koch et al. 2006). Interestingly, Byrne (2008) found similar results when reviewing Australian biota refugia but had different explanations.

We are fully aware that the explanation for the results and patterns we obtain is narrative. However, as this is the first phylogeographic study of a southern African thicket floristic element, our results emphasize the urgent need for more studies on other thicket elements.

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