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THE PHYLOGENY OF THE *PENTASCHISTIS* CLADE (DANTHONIOIDEAE, POACEAE) BASED ON CHLOROPLAST DNA, AND THE EVOLUTION AND LOSS OF COMPLEX CHARACTERS

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We construct a species-level phylogeny for the *Pentaschistis* clade based on chloroplast DNA, from the following regions: trnL-F, trnT-L, atpB-rbcL, rpL16, and trnD-psbA. The clade comprises 82 species in three genera, *Pentaschistis*, *Pentameris,* and *Prionanthium*. We demonstrate that *Prionanthium* is nested in *Pentaschistis* and that this clade is sister to a clade of *Pentameris* plus *Pentaschistis tysonii*. Forty-three of the species in the *Pentaschistis* clade have multicellular glands and we use ancestral character state reconstruction to show that they have been gained twice or possibly once, and lost several times. We suggest that the maintenance, absence, loss, and gain of glands are correlated with leaf anatomy type, and additionally that there is a difference in the degree of diversification of lineages that have these different character combinations. We propose that both glands and sclerophyllous leaves act as defense systems against herbivory, and build a cost/benefit model in which multicellular glands or sclerophyllous leaves are lost when the alternative defense system evolves. We also investigate the association between leaf anatomy type and soil nutrient type on which species grow. There is little phylogenetic constraint in soil nutrient type on members of the *Pentaschistis* clade, with numerous transitions between oligotrophic and eutrophic soils. However, only orthophyllous-leaved species diversify on eutrophic soils. We suggest that the presence of these glands enables the persistence of orthophyllous lineages and therefore diversification of the *Pentaschistis* clade on eutrophic as well as oligotrophic soils.

KEY WORDS: **Ancestral, correlated evolution, Danthonioideae, gland evolution, *Pentaschistis*, phylogeny, Poaceae.**

Interest in the evolution of complex structures and their influence upon the success of organisms has a long history. Classic examples include orchid flowers (Darwin 1862) and the evolution of eyes (Fernald 2004). Part of their fascination lies in the very low probability that such structures should evolve at all (Dawkins 1986). However, their (often) unique evolution makes it difficult to tease apart the adaptive aspects from other factors, including chance. In contrast, the loss of such complex structures presents us with an opportunity to explore their function. Losses tend to be more numerous than gains in many complex structures or traits (e.g., heterostyly [Kohn et al. 1996; Schoen et al. 1997]; secondary xylem in aquatic plants [Sculthorpe 1967]; wings in insects [Whiting et al. 2003]; or the chlorophyll producing function of chloroplasts, in holoparasites [Judd et al. 2002; APG 2003; Bungard 2004]; Nickrent et al. 2004]). This makes them more tractable to investigation than gains. For example, if the losses of a particular structure within a clade are nonrandom with respect to other morphological characters or habitat, these losses can be used to investigate

the function of this structure. Here we investigate the evolution of multicellular glands in the *Pentaschistis* clade in this context.

Peculiar multicellular glands (see Fig. 1A, B) exist on the inflorescences and/or leaves in 43 species of *Pentaschistis* and of *Prionanthium*, and are formed from groups of cells, of which some are secretory. These glands are unique in the grasses, developing from the epidermal cells, rather than from preexisting bicellular hairs on the epidermis (Davidse 1988; Linder et al. 1990). They range from simple linear glands consisting of enlarged epidermal cells arranged in a series, to more elaborate glands that have a greater size differentiation between glandular and epidermal cells, and further some that have differentiated cell types within the gland. Equating the elaborate type with a derived condition (and "simple" as plesiomorphic), evolutionary progression from simple to complex glands was suggested following morphological–anatomical analysis (Linder et al. 1990). However, neither the evolutionary progression nor the number of times that glands were gained or lost has been thoroughly investigated. To date no species-level phylogenetic hypothesis of the clade exists. The current infrageneric classification of *Pentaschistis* was based on morphological data and was constructed to aid communication and identification, not to reflect phylogeny (Linder and Ellis 1990). To understand the evolution of these glands, however, a historical, phylogenetic component is vital (Felsenstein 1985b; Pagel and Harvey 1988), preferably one based on characters other than those being studied (Coddington 1988; Armbruster 1992; but see Luckow and Bruneau 1997).

The *Pentaschistis* clade (Barker et al. 2000) comprises *Pentaschistis* (Nees) Stapf with ~70 species, *Pentameris* Beauv. with nine species, and *Prionanthium* Desvaux with three species, and is the most species-rich group of grasses in the Cape flora of South Africa. Although *Pentameris* and *Prionanthium* are both endemic to the Cape Floristic Region (CFR, Goldblatt 1978), *Pentaschistis* is widespread in temperate habitats in sub-Saharan Africa and is a frequent element in the Afromontane and Afroalpine vegetation (Knapp 1973). Within the CFR, most of the species are found in a variety of habitats in fynbos vegetation across wide altitudinal and rainfall regimes. They exist mostly not only on soils derived from sandstones of the Cape Supergroup, but also on the limestone hills of Bredasdorp (e.g., *Pentaschistis calcicola*), coastal sands (e.g., *Pentaschistis barbata*), shale in coastal Renosterveld (e.g., *Prionanthium ecklonii*), and silcrete derived soils (e.g., *Pentaschistis juncifolia*).

This essentially south-temperate group (Linder 1989) is taxonomically well known (Linder and Ellis 1990; Phillips 1994; Galley and Linder 2006). The cytology of many species has been investigated, all three genera have a basic chromosome number of x = 7 (Davidse 1988; du Plessis and Spies 1992; Spies and Roodt 2001). Comprehensive investigation of the leaf anatomy of almost all species in the clade has been carried out (Ellis 1989; Ellis

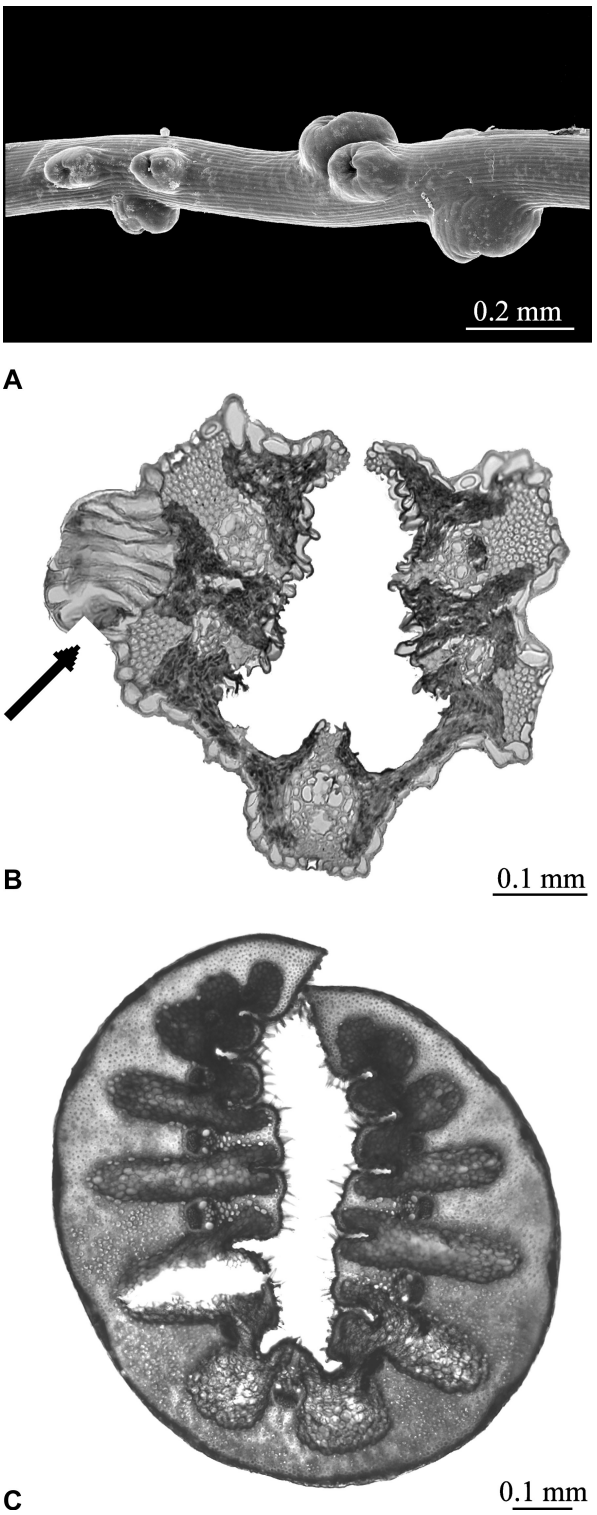


Figure 1. **(A) Stalked multicellular gland from *Pentaschistis airoides* subsp. *jugorum*; (B) transverse section (TS) of a leaf of *Pentaschistis clavata*, illustrating the orthophyllous anatomy type with noncontinuous girders (pale gray) and mesophyll tissue (dark gray), also a multicellular gland (arrow); (C) TS of a leaf of *Pentashistis horrida*, illustrating the sclerophyllous anatomy type with extensive, continuous girders (pale gray) and restricted mesophyll tissue (dark gray).**

Table 1. Characteristics of sclerophylls and orthophylls, adjusted from Ellis and Linder (1992).

Orthophyllous leaves	Sclerophyllous leaves
Diffuse mesophyll of large rounded parenchyma cells	Compact mesophyll of small isodiametric cells
Extensive intercellular air space system	Minute intercellular air spaces
Sclerenchyma only associated with the bundles as girders or strands	Sclerenchyma abundant—often continuous between bundles (via the hypodermis)
Cuticle and outer walls of epidermal cells thin	Cuticle plus outer epidermal cell wall thickened
Abaxial stomata present	Abaxial stomata absent
Epidermal zonation evident (abaxial)	No distinction between costal and intercostal zones in surface view

and Linder 1992; Barker 1993) allowing a broad classification of the species into two types, those with orthophyllous leaves and those with sclerophyllous leaves (Ellis and Linder 1992). Micromorphological characteristics used to define these leaf types are shown in Table 1 and illustrated in Figures 1B and 1C. Orthophyllous leaves tend to have soft blades and a life expectancy of at most one growing season, whereas sclerophyllous leaves are thick and tough, generally living for more than one growing season (Ellis and Linder 1992).

The evolution and function of the glands, however, remain a puzzle, and we seek to account for why some species have these complex glands whereas others do not. We reconstruct a species-level phylogeny of the *Pentaschistis* clade based on plastid DNA sequence data to resolve phylogenetic relationships within the clade, and to determine how many times glands evolved and have been lost. It is evident that there is a relationship between the presence of these glands and leaf anatomy type within the clade: glands are more common in orthophyllous taxa than in sclerophyllous taxa (Ellis and Linder 1992). The evolution of these glands is therefore most productively addressed in relation to leaf anatomy type. We use ancestral character state reconstruction to investigate the frequency with which character combinations evolve and are retained, and also compare lineage diversification between these character combinations. Finally, we seek to relate leaf anatomy type and soil nutrient type.

Materials and Methods

SAMPLING

We attempted to obtain complete species sampling for the *Pentaschistis* clade, based on the taxonomy of Davidse (1988), Linder and Ellis (1990), and Barker (1993), as well as recently described species and taxonomic changes (Phillips 1994, 1995; Galley and Linder 2006). Seventy-three of 82 species were obtained, representing 80 of 90 taxa, including the four varieties of *Pentaschistis pictigluma* and both subspecies of *P. airoides* and *P. aurea*. Based on the subfamily phylogeny of Barker et al. (2000), two *Merxmuellera* species were selected as outgroups. Material was collected into silica gel in the field, except for material from three species that was obtained from herbarium specimens (see Appendix for details).

MOLECULAR TECHNIQUES

Tissue collection and DNA isolation

DNA was extracted using DNeasy Plant Mini Kits (Qiagen, Switzerland) following the manufacturer's protocol or the CTAB method (Doyle and Doyle 1987). Where DNA was extracted from herbarium material, a modification of the SDS method (Eichenberger et al. 2000) of DNA extraction was used, repeating the chloroform-cleaning step.

PCR amplification

Polymerase chain reactions (PCRs) were performed in a Biometra Thermocycler TGradient (Biometra, Göttingen, Germany) using a total reaction volume of 25 μL with 2.5 mM $MgCl_2$, 1 × PCR buffer (Amersham Biosciences, Otelfingen, Switzerland), 0.25 mM dNTPs, 1.6 μM primer, and 1 unit of Taq polymerase (Amersham Biosciences, and Sigma-Aldrich, Buchs, Switzerland). Additives were used as described. The intergenic spacers *trnT-L, L-F, atpB-rbcL*, and *trnD-psbA* and introns of *trnL* and *rpL16* were amplified by the PCR method and sequenced using the primers as shown in Table 2.

The *trnL-F* intergenic spacer and *trnL* intron (trnLF) were amplified using a PCR protocol with an initial denaturation step of 94°C for 3 min followed by a step-down program with four cycles of 1 min denaturation at 94°C, 1 min annealing at 56°C, 1 min extension at 72°C, then 28 cycles of 1 min at 94°C, 1 min at 52°C, and 1 min at 72°C, terminated by a final extension of 7 min at 72°C. The *trnT-L* intergenic spacer (trnTL) was amplified using a similar program, but using an annealing temperature of 54°C in the first part of the step-down section. One microliter of BSA was used in all reactions. The *atpB-rbcL* intergenic spacer (atpB-rbcL) was amplified using a similar program with the step down as follows: three cycles of 1 min at 95°C, 1 min at 55°C, 1 min at 72°C, then six cycles of 1 min at 95°C, 1 min at 52°C, 1 min at 72°C, then 26 cycles of 1 min at 94°C, 1 min at 48°C, and 1 min at 72°C. A total of 1.2 μL DMSO or 4 μL of BSA were used for several samples that would otherwise not amplify. The *rpL*16 intron (rpL16) was amplified using an initial denaturation step of 94°C for 4 min followed by 34 cycles of 1 min

Table 2. Primers used for PCR and sequencing.

Gene region	Primers (PCR)	Primers (sequencing)	Source	Sequence (if from this study)
trnL-F	c,f	c,d,e,f,	Taberlet et al. 1991	
		c2	this study	5′-GGT CCT YAA ACT ARA ACC C-3′
		d3	this study	5′-GKG KMT RGT ATT ATA TCC-3′
trnT-L	a,b	a,b	Taberlet et al. 1991	
atpB-rbcL	f1c, r1a2	f1c, r1a2	Hardy and Linder 2005	
		atpBrbcL_Fint	this study	5′-GTG TAY TGK ACR TTC TA-3′
		atpBrbcL_Rint	this study	5′-CCR AAA WYC CAA ARG CCA-3′
rpL16	F71	F71	Baum et al. 1998	
	R1000	R1000	this study	5′-CTG TTC TTT TRG GTT ATA GTC-3′
trnD-psbA	trnD	trnD	Shaw et al. 2005	
	psbM	psbM	Shaw et al. 2005	5′-TAG AGT WCC MRT ATT TTA CCG-3′
		trnCD3	this study	5′-CTA GGG TTC AAT GAA TGG-3′
		trnCDF2	this study	

at 94°C, 1 min at 55°C, and 1 min 20 sec at 72°C, terminated by a final extension of 7 min at 72°C. One microliter BSA was used in each sample. The *trnD-psbA* intergenic spacer (trnD) was amplified using an initial denaturation step of 94°C for 3 min followed by 30 cycles of 1 min at 94°C, 1 min at 52°C, and 1 min 40 sec extension at 72°C, terminated by a final extension of 7 min at 72°C. One microliter of BSA was used for several samples that would otherwise not amplify. All PCR products were visualized on a 1.5% agarose gel. Double bands were found for some accessions for trnD. In these cases the band of appropriate size (between 1100 and 1200 base pairs) was excised and purified with a DNA band purification kit (Amersham Biosciences) before sequencing.

Sequencing

PCR products were cleaned using the QIAquick PCR purification kit (Qiagen, Basel, Switzerland) according to the manufacturer's protocol, and cycle sequencing was carried out on an ABI Prism, LA, USA 3100 Genetic Analyzer (Applied Biosciences, Foster City, CA, USA) using BigDye terminator versions 2.0 and 3.1 without and with 5 × buffer (Applied Biosciences), respectively.

PHYLOGENETIC ANALYSIS

Data matrices were aligned by eye, and gaps were coded by hand using the nested gap principle of Simmons and Ochoterena (2000), except for gaps created by poly A or poly A/T regions and gaps that represented single base pairs, which were not coded. Datasets were analyzed separately and in combination using the parsimony criterion, by means of the Ratchet implemented in Winclada (Nixon 1999–2002). Default settings were used, calculating 12 consecutive runs. Bootstrap support (BS; Felsenstein 1985a) was also calculated in Winclada using 500 bootstrap replicates with 50 replicates per bootstrap replicate, holding three trees per replicate. Potential incongruence between datasets was assessed visually by comparing (1) phylogenies from individual datasets, and (2) the support and resolution of phylogenies from the combined data versus individual gene regions. There was no incongruence with BS above 50%. The combined "chloroplast" dataset resulted in a more highly resolved phylogenetic tree with greater support for the groupings than did the individual datasets, and was used for final phylogenetic analysis.

The chloroplast dataset was additionally analyzed with a likelihood criterion using Bayesian analysis implemented in MrBayes 3.0 beta 4 (Huelsenbeck and Ronquist 2001). The dataset was separated into seven partitions (rpL16, trnTL, trnD, trnLF, atpB-rbcL, the poly-AT regions, gapcoding). Poly-AT regions and gap coding from all gene regions were pooled to form the "poly AT" and "gaps" partitions, respectively. The seven partitions were analyzed individually or combined, to test whether pooled partition "schemes" might yield a better fitting model. Each partition received its own model in the seven-partition scheme, the five gene regions (excluding poly AT data) were merged in the three-partition scheme, and all DNA characters were merged in the two-partition scheme (see Table 3). Each partition scheme (except for "gapcoding," which was coded as "standard" data for the phylogenetic analysis) was assigned a model using Modeltest (Posada and Crandall 1998) implemented in PAUP* (Swofford 2002), using the Akaike Information Criterion (AIC, Akaike 1973) to choose between models. Information from the model (Nst, gamma, and presence of invariant sites) was then used in the Bayesian analysis, but the parameter values were free to vary. Each of the three partition schemes was analyzed using Bayesian phylogenetic analysis with four chains (three hot, one cold) run for 4,000,000 generations sampling every 1000 generations. The likelihood values

Table 3. Partition schemes tested in Bayesian analysis and results from the AIC test.

	Partitions	Parameters	lnL of the harmonic mean	AIC	ΔAIC
All DNA (1) gaps (1)	2	14	−22283.96	19,389.30	243
gene regions (1) poly AT (1) gaps (1)	3	26	−21957.59	19,145.96	0
gene regions (5) poly AT (1) gaps (1)	7	62	−21978.24	19,483.68	338

of the sampled models were checked in previous shorter analyses (500,000 generations) to obtain burn-ins of 170,000 (two and three partition analysis) and 250,000 (seven partition analysis) generations. The likelihood of each parameter was checked for stability, indicating convergence.

The harmonic mean of log likelihoods (lnL) of all generations (excluding burn-in) was used to compare the three partition schemes, as this is less sensitive to outliers than the mean lnL (Nylander et al. 2004). The AIC was used to compare these harmonic means as it penalizes high-parameter models (Burnham and Anderson 2004). The three-partition model had an AIC score that was 243 units higher than the next competing model set (see Table 3), a score considered to provide strong evidence (Burnham and Anderson 2004). Consequently the three-partition model was used for further analyses.

We checked three subsequent runs for the convergence of topology and likelihood scores. From all the runs, the generation that received the overall highest likelihood score was used as a "final topology." This overall likelihood score is a combination of all parameters of which the topology is just one. However, a fully resolved topology was preferred for ancestral state reconstruction, and we find this method of choosing a topology superior to randomly picking one. A 50% majority rule of the set of sampled trees (excluding burn-in trees) was constructed to obtain posterior probabilities (p.p.) as a measure of node support. Nodes that were not in this 50% set were treated as described below.

Following rejection of a molecular clock ($df = 86$, $P < 0.01$), branch lengths were made ultrametric using Penalized Likelihood in r8s (Sanderson 2002, 2003) using the smoothing parameter 0.00126, yielded from cross validation.

DESCRIPTION AND SCORING OF GLANDS AND LEAF ANATOMY TYPE

Glands were scored as either absent, linear, or rounded, based on personal observation and literature (Davidse 1988; Linder and Ellis 1990; Linder et al. 1990; Barker 1993). Linear type glands were scored as such, and the club shaped, sessile elongated, and sunken crateriform glands (see Linder et al. 1990) were scored as rounded. We did not consider the position of the glands on the plant, assuming that their function is independent of their position.

Data on leaf anatomy type were mostly derived from the literature (Ellis 1989; Ellis and Linder 1992; Barker 1993), and species were classified as sclerophyllous or orthophyllous, in accordance with Ellis and Linder (1992). The leaf anatomy type is inadequately known for *Pentaschistis andringitrensis* A. Camus, *P. insularis* (Hemsl.) Linder, *Pentaschistis clavata* Galley, *P. horrida* Galley, and *P. trifida* Galley. Hand- and microtome-sections were prepared from herbarium specimens for these species. For the hand sections, midsections of dried leaves were softened in boiling soapy water for 10–15 min, hand-sectioned, and stained in a 1:1 mix of safranin red/alcian blue (Tolivia and Tolivia 1987) for 5–10 min. After rinsing in water the sections were dehydrated in ethanol and mounted with histoclear and histomount. For the microtome sections, midsections of dried leaves were softened in sulfosuccinate sodium salt and acetone for 2–5 h, plastic embedded in GMA (Igersheim and Cichock 1996), and sectioned to 6–8 μm thick. Sections were stained in toluidine blue (3–4 min) and ruthenium red (1–2 min) and mounted with histoclear and histomount. Leaf anatomy type was assigned according to Ellis and Linder (1992) using the characters shown in Table 1.

SCORING OF SOIL NUTRIENT TYPE

Each species was assigned to either eutrophic or oligotrophic soil type based on the bedrock or substrate type on which the species grows. This information was obtained from herbarium specimen label data (BOL and Z collections) and from field observations. Eutrophic soil type was assigned to soils derived from shale, coastal sands, granite, silcretes, and basalt, and oligotrophic soil type was assigned to soils derived from sandstones of the Cape Supergroup (CS), limestone (pavements), cave sandstone, and well-leached (acid) sands. Soil fertility is difficult to quantify, dependant not only on the nitrogen (N) and phosphorus (P) status of the soils, but also on their availability. We base our assignments on literature (Killick 1979; Kruger 1979; Lambrechts

1979; Witkowski and Mitchell 1987) using *P* and, where possible, N levels as guides. The method of likelihood optimization that we used does not allow polymorphic taxa. In the few cases that a species is recorded from both eutrophic and oligotrophic soils, it was scored only as oligotrophic, as such nutrient constraints would be expected to be a limiting factor for the plants.

CHARACTER OPTIMIZATION

A likelihood approach was used for ancestral character state reconstruction, which enables the rate-corrected branch lengths to be taken into account. More importantly, it provides an estimate of the uncertainty in the reconstruction (Schluter et al. 1997), which is especially relevant for nodes deep in the phylogeny. Branches receiving less than 0.50 p.p. were shortened to a length of 0.0001. All nodes were optimized in Mesquite version 1.11 (Maddison and Maddison 2006). A two-rate model offered no significant improvement in likelihood for leaf anatomical type, so following Mooers and Schluter (1999), a one-rate model was used. A two-rate model did offer significant improvement in likelihood for soil fertility and gland presence and was used for these characters. A difference of 2 log likelihood units (lnL) between character states for a given node was considered as statistically significant (Pagel 1994, 1999). This corresponds to ~0.89 proportional likelihood. Nodes that could not be optimized with statistical significance were omitted from calculations. The exception to this is soil fertility in which most nodes had a difference of slightly below 2 lnL. Here we used a proportional likelihood value of 0.80 as a threshold for statistical significance (see Results). In all cases outgroups were removed from the phylogenetic tree and the tree was rooted at the node between the *Pentameris* sensu lato clade and *Pentaschistis*/*Prionanthium* clade.

Taxa were scored with binary coding and all internal nodes were optimized for glands as present or absent, linear or not linear (i.e., no glands, or rounded glands), and rounded or not rounded (i.e., no glands or linear glands); for leaf anatomy type as orthophyllous, sclerophyllous; and for soil type eutrophic, oligotrophic.

ASSOCIATIONS BETWEEN CHARACTERS

A modification of the contingent states test (Sillén-Tullberg 1993; Werdelin and Tullberg 1995) was used to test for the association between character states, as this test allows both the maintenance and the change in character states to be considered separately. For each node two "events" are counted, that is, from the node to each of the daughter lineages (Sillén-Tullberg 1993). For each event the maintenance or change in character state for both characters is scored as illustrated in Figure 2. There was one case of topological uncertainty, which might alter the changes inferred, depending on which way the nodes are resolved. Although this does not alter the overall conclusions we reach, the changes to and from this node were omitted from the counts (the node

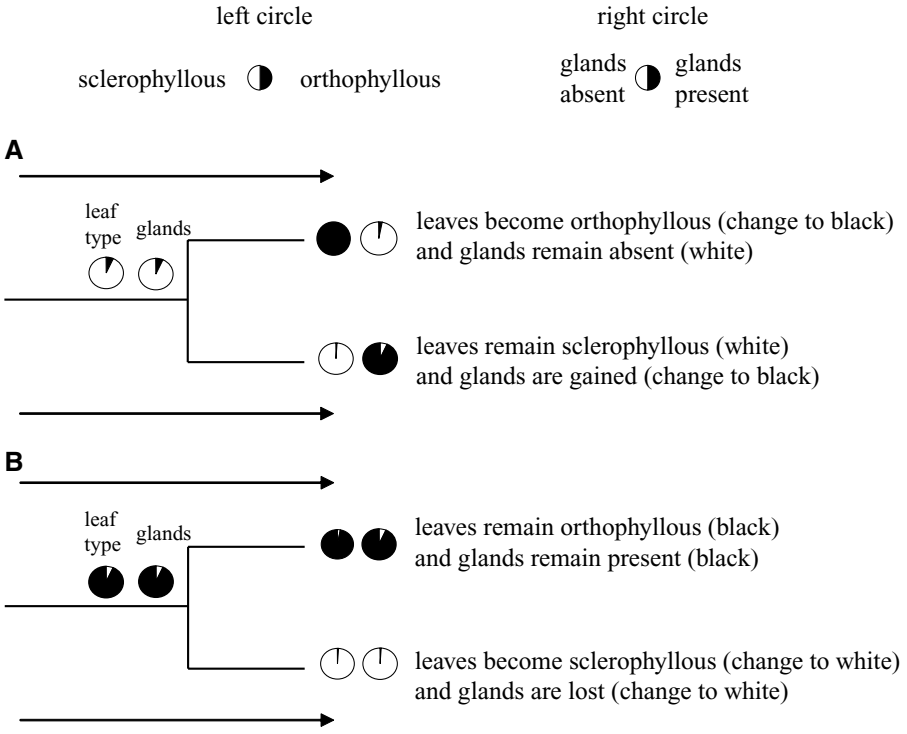


Figure 2. **Illustration of how "events" are counted at each node according to the contingent states test (Sillén-Tullberg 1993). Leaves may change from sclerophyllous to orthophyllous or remain sclerophyllous (A) or they may persist as orthophyllous or change to sclerophyllous. (B) Likewise leaves may gain or lose glands, or remain in the current state. Sixteen types of events are possible.**

subtending the group *P. cirrhulosa* and *P. calcicola*). To examine the association between gland evolution and leaf anatomy type we scored the number of cases of maintenance of glands, maintenance of no glands, evolution of glands, and loss of glands, against two leaf types: orthophyllous, including transformation from sclerophyllous to orthophyllous, and sclerophyllous, including transformation from orthophyllous to sclerophyllous. To examine the association between leaf anatomy type and soil nutrient type, we tested the different leaf states (as above) on oligotrophic, including change from eutrophic to oligotrophic, and eutrophic, including change from oligotrophic to eutrophic soil types. The soil types were then decomposed to investigate the diversification associated with a transition between soil types and diversification within each soil type. A G-test using Yates' correction for small values was used to test the significance of these associations (Sokal and Rohlf 1995). In several cases multiple accessions were left in the phylogeny to represent the different lineages, but for the contingent states tests one accession of *P. natalensis* and *Pentaschistis colorata* each was omitted.

Results

PHYLOGENY RECONSTRUCTION

A matrix with a total of 5479 aligned characters, combining DNA sequence data and 56 gap characters from five data partitions representing four chloroplast regions, was analyzed. The number of parsimony informative characters for atpB-rbcL was 103; for rpL16, 104; for trnLF, 128; for trnTL, 171; and for trnD, 126. There are no nodes in the strict consensus of the most parsimonious trees that conflict with the 50% majority rule set of Bayesian trees. There is a general positive relationship between the BS and Bayesian p.p. values, but some nodes that have 1.00 Bayesian p.p. values have a wide range of BS values.

Clade names have been allocated to groups in the phylogeny for convenience, and are named as the first species to branch off within the group. *Pentameris* plus *P. tysonii* form a clade (*Pentameris* sensu lato clade, I) that is sister to the remainder of the *Pentaschistis* clade including *Prionanthium* (see Fig. 3), the "*Pentaschistis*/*Prionanthium* clade." The monophyly of each of these clades is strongly supported. Within the large *Pentaschistis*/*Prionanthium* clade, the *P. elegans* clade (II) is sister to the rest of the group. The next species to branch are *Pentaschistis basutorum*, *P. juncifolia*, and *P. andringitrensis*, forming a grade. The remaining species form a well-supported clade containing almost all of the glanded taxa. The first clade to branch within this is a clade comprising the *P. argentea* clade (III) and the *P. aurea* clade (IV), although the support for this sister relationship is weak. The *P. ampla* and *P. exserta* species pair are next to branch. The two *Prionanthium* species sampled are sister to the *P. rosea* group and this strongly supported clade (V) is the next clade to diverge. Two species pairs and *P. triseta* form a grade that diverges basally to a well-supported clade that comprises a "coastal clade" (VI) and a large "summer rainfall clade" (VII). The "coastal clade" comprises two noncoastal species (*P. reflexa* and *P. rupestris*), but the core group is distributed near the coast, albeit growing on soils derived from a variety of rock types. The "summer rainfall clade" contains species occurring in the drier areas (*P. lima*, *P. aristifolia*, *P. tomentella*, *P. airoides* ssp. *airoides*, *P. pseudopallescens*, *P. veneta*, and *P. montana*) but also most of the summer rainfall species. These include several species distributed in the Drakensberg (South Africa), a polyphyletic *P. natalensis* ranging from the Drakensberg north toward southern Tanzania and east to Madagascar, and the taxa that exist exclusively in the mountains of tropical Africa.

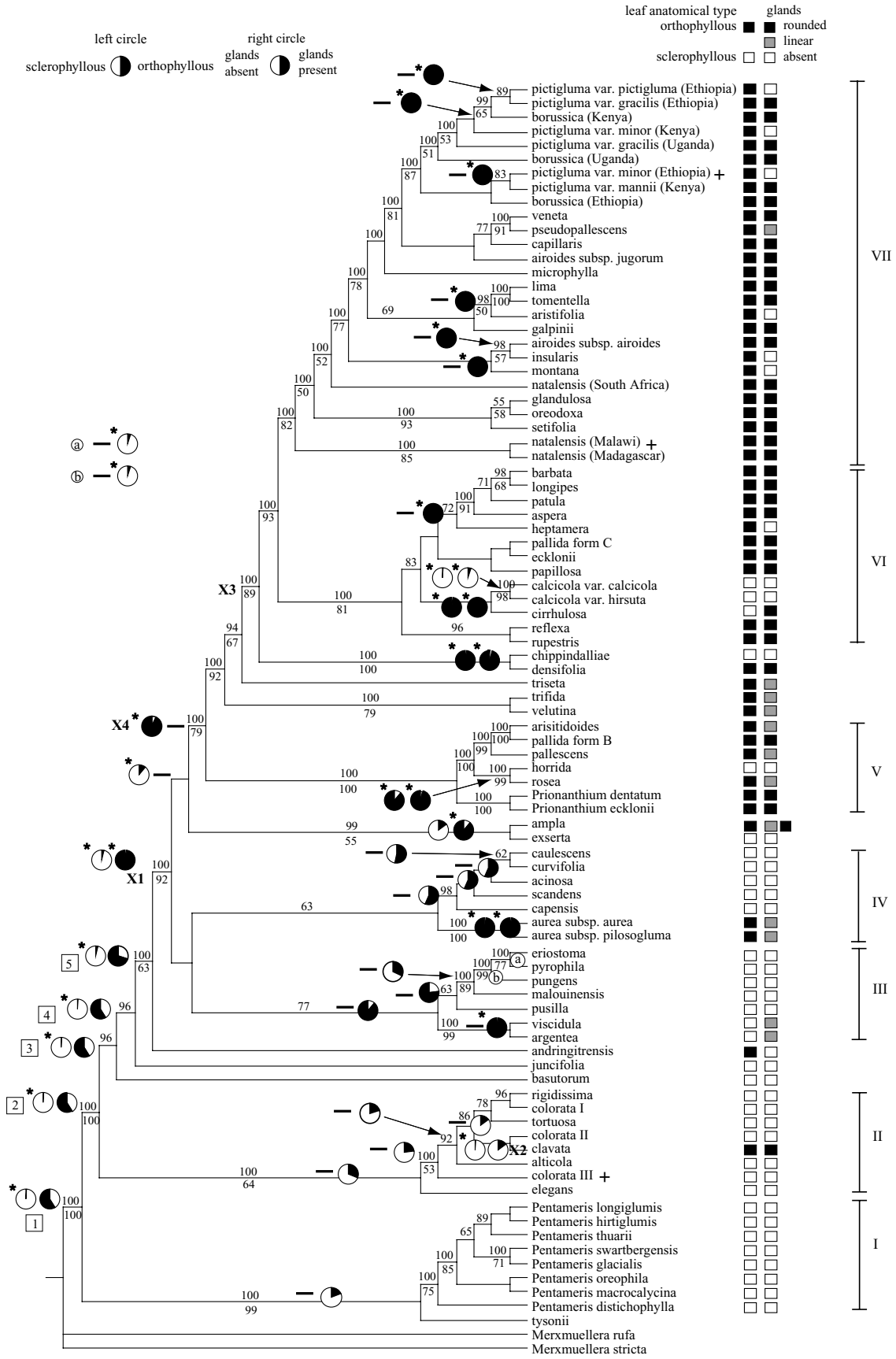
EVOLUTION OF GLANDS AND LEAF ANATOMY TYPE

We classify the leaf anatomy type of *P. clavata*, *P. andringitrensis*, *P. insularis*, and *P. trifida* as orthophyllous, and *P. horrida* as sclerophyllous.

In the ancestral character state reconstruction of gland presence/absence, most nodes were unambiguously assigned a character state, except some nodes along the spine of the phylogeny (see Fig. 3, nodes numbered 1–5 in square boxes) and other nodes as shown (see Fig. 3). These nodes were omitted from further calculations because the likelihood values between character states for the glands and/or leaf anatomy type were almost equivocal.

Glands evolved twice in the *Pentaschistis* clade: once in *P. clavata* and once at the base of a larger clade comprising at least 58 species (see Fig. 3, X2 and X1, respectively). At node X1 there is strong evidence of glands being present (difference in lnL of 3.90), whereas the node preceding this is much less decisive (differences in lnL between 0.88 and 0.40). There are nine nodes between X1 and *P. clavata*. Forcing the optimization to a single gain of glands would require eight losses of glands in the eight diversification events between them. Based on our sampling, the most likely hypothesis is therefore that glands evolved twice: once as rounded glands (*P. clavata*) and once as linear glands (Fig. 3, X1). Rounded glands evolved from linear glands four times, including *P. ampla*, *Prionanthium*, *P. pallida*, and by node X3 (see Fig. 3), but there is only a single transition in the reverse direction (*P. pseudopallescens*). There are several losses of glands (see Fig. 4), from both linear and rounded glands. Unequal rates of gland gain and loss are also demonstrated by a likelihood analysis. The difference between the single-rate and the two-rate models for glands is significant (difference in lnL = 4.29), which shows that the difference between the rates of gland gain (0.54) and loss (3.50) is significant.

There is a significant bias in the distribution of glands relative to leaf type (G-test, df = 1, $P < 0.0001$). Glands are more



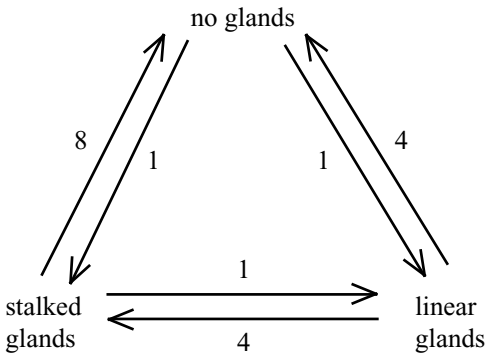


Figure 4. Number of changes between the gland states.

frequently found in species that have orthophyllous leaves, whereas eglandular species tend to have sclerophyllous leaves.

In both cases glands evolved near or at a change from sclerophyllous to orthophyllous leaf type, and a total of 12 gland losses occur in the *Pentaschistis* clade (the losses in the two geographic lineages of *P. pictigluma* var. *minor* are counted as one event). The maintenance of a glandless state is higher in a background of sclerophyllous leaves than orthophyllous leaves (Table 4). Some of this bias derives from the evolution of glands in a part of the lineage in which few of the sclerophyllous species exist. However, this bias remains if we consider species within the glanded clade only; three of the sclerophyllous lineages that have lost glands diversify representing 12 taxa, and these lineages all remain glandless. In contrast to this, none of the six orthophyllous lineages that lose glands diversify. There is a strong bias in gland maintenance and gland loss between the different leaf types (see Table 4; G-test, $df = 1$, $P = 0.003$), and the ratios of gland maintenance to gland loss are approximately 18:1 for orthophyllous lineages and 1.5:1 for sclerophyllous lineages. This demonstrates that glands tend to be kept rather than lost in orthophyllous lineages. There are only three taxa that have both glands and sclerophyllous leaves, but this represents only one diversification event.

In sum, the two character combinations, sclerophyllous leaves without glands and orthophyllous leaves with glands, are associated with a high number of diversification events. In contrast, the two character combinations, sclerophyllous leaves with glands and orthophyllous leaves without glands, are associated with few or no diversification events, respectively.

There are nine transitions between orthophyllous and sclerophyllous leaf type in the *Pentaschistis* clade (see Fig. 3). There are four transitions from orthophyllous to sclerophyllous leaf type, three of which are accompanied by the loss of glands.

SOIL NUTRIENT STATUS AND LEAF ANATOMY

The earliest diverging members in the *Pentaschistis* clade tend to exist on oligotrophic soils. However, node optimization for soil nutrient type was rarely decisive, especially at the basal nodes (see Fig. 5), a phenomenon probably caused by the relatively low grouping information of the character (R.I. = 0.46 compared with glands R.I. = 0.65 and leaf anatomy type R.I. = 0.80).

The balance of likelihood scores of character states along the "spine" of the phylogeny shows the evolution of orthophyllous leaves from sclerophyllous leaves by node X4 (see Figs. 3 and 5), also change from oligotrophic to eutrophic soils (see Fig. 5). There is a strong bias in the occurrence of different leaf types on eutrophic and oligotrophic soils (see Table 5, in bold type; G-test $P < 0.0001$, $df = 1$); sclerophyllous plants exist almost exclusively on oligotrophic soils, whereas orthophyllous plants exist more or less equally on both soil types. This can be decomposed to identify changes or maintenance of states at nodes, as shown in Table 5. Although sclerophyllous and orthophyllous taxa evolve onto eutrophic soils a more or less equal number of times, the bias between leaf anatomy type and soil nutrient type occurs because there is no diversification of sclerophyllous lineages on eutrophic soils (a conclusion that is upheld even if the complete phylogeny is considered). Conversely, orthophyllous lineages diversify on both soil types, although the diversification rate (per change

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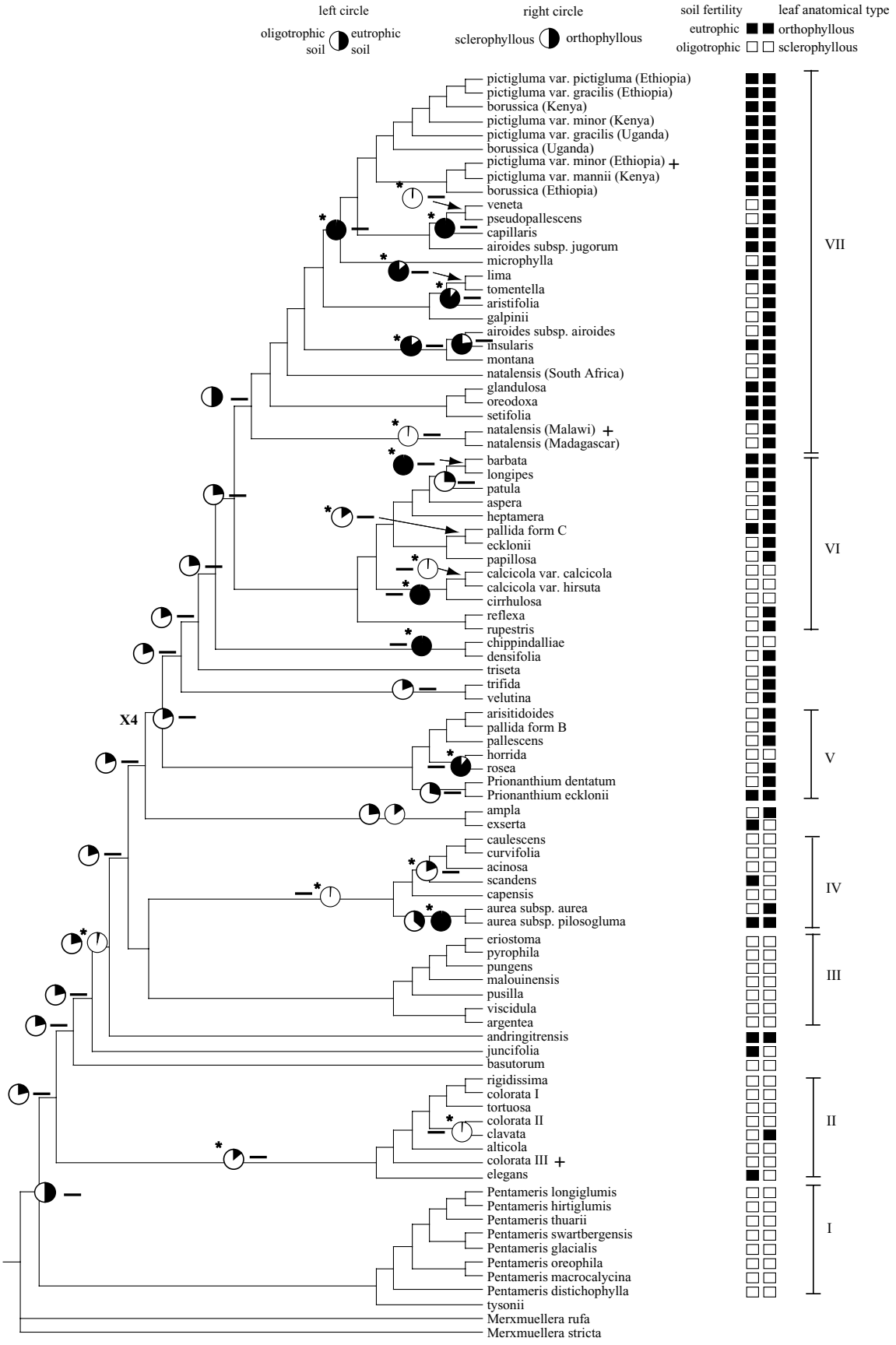
Figure 3. Phylogenetic hypothesis from Bayesian analysis showing the topology from the most likely model, with posterior probabilities from the Bayesian analysis (> 50 support) shown above branches (probabilities 0 to 1 converted to scores 0 to 100) and bootstrap support (BS) shown below branches (> 50% support). The left column of squares shows taxa as orthophyllous or sclerophyllous and the right column of squares shows taxa as having no glands, linear glands, or rounded glands. Pie charts show optimization (as proportional likelihoods) at nodes that subtend gains/losses in glands, and nodes that are not unambiguously optimized. Pie charts are marked with an asterisk (upper left side) when optimization is statistically significant. Dashes are placeholders for pie charts that are not shown. Node X1 and tip X2 indicate points from which glands have evolved. Node X3 is where the rounded glands are reconstructed as having evolved from linear glands. Unless otherwise illustrated, all nodes within this glanded clade (X1) were optimized with statistical significance as glands "present," and all nodes outside it as glands "absent." Unless otherwise illustrated, all nodes in clade X4 were optimized with statistical support as orthophyllous-leaved and nodes outside this clade as sclerophyllous. Accessions marked with a plus sign and nodes numbered in square boxes were omitted from the contingent states test (see text). Clade names (I to VII) are informal names referred to in the text. Unless otherwise indicated, all species are *Pentaschistis*.



onto each soil type) is higher for eutrophic soils (approximately 18 diversification events per change for eutrophic soils vs. approximately four diversification events per change for oligotrophic soils). Within the "coastal clade" and the "summer rainfall clade" especially, lineages change frequently between soil type and diversify on both.

Discussion

We used DNA sequence data from four chloroplast regions and derived gap characters to reconstruct a phylogenetic hypothesis for the *Pentaschistis* clade. Both parsimony and Bayesian analysis provide moderately well-resolved and well-supported phylogenetic hypotheses with some exceptions, clustered in the spine (see Fig. 3). Analyses of individual datasets lacked well-supported resolution in this region, suggesting that lack of characters rather than conflict between datasets causes low resolution in these parts of the phylogeny. This is reflected in the very short branch lengths around these nodes in the individual analyses (data not shown).

We used a fully resolved phylogeny for ancestral character state reconstruction. We minimized the influence of branches that received less than 0.50 p.p. by giving them very short branch lengths. Also, in many cases, nodes with low support could not be ambiguously optimized and were therefore omitted from further analysis. From inspection, there was only one region in the topology in which different node arrangements would have altered the changes counted between character states, and the node here was also omitted from the counts. Otherwise, most topological uncertainty was in terminal branches and most changes in soil type and gland losses, especially, are fairly well dispersed throughout the phylogeny.

SYSTEMATICS

The *Pentaschistis* clade was named as such following phylogenetic analysis of DNA sequence data of the Danthonioideae by Barker et al. (2000). The grouping of these three genera is also supported by morphological, histological, and cytological features. These include the secondary loss or weak development of haustorial synergids, fine granular, or no starch in the synergids (compared to globular starch in other Danthonioid genera; Verboom 1994), the insertion of the lemma setae in the sinuses between the lateral lobes and the median awn, although this is also found in *Pseudopentameris* (Verboom and Linder 1997), a basic chromosome number x = 7 (Spies and Roodt 2001) and palea veins that do not reach the tip of the palea.

The close relationship between *Pentaschistis* and *Pentameris* (Clayton and Renvoize 1986) has long been recognized. *Pentameris* was described by Palisot de Beauvois (1812) on account of four bristles (presumably two lemma lobes and two bristles), the central awn of the lemma, and the shape and hairiness of the seed. The name *Pentaschistis* was first used 29 years later for a segregate of *Danthonia* (Nees ab Esenbeck 1841). The distinction between *Pentaschistis* and *Pentameris* has not always been clear and species were often transferred between the two genera (Kunth 1833; Nees ab Esenbeck 1841; Durand and Schinz 1895) or were considered in the same taxon (Durand and Schinz 1895). Stapf (1899) defined *Pentameris* based on the structure and hairiness of the ovary, recognizing a total of five species. The two genera have since remained separated (e.g., McClean 1926; Barker 1993). This distinction is supported by our results, with the exception of *P. tysonii* that is sister to *Pentameris*.

In contrast with *Pentameris*, the close relationship between *Prionanthium* and *Pentaschistis* has only recently been recognized. When *Prionanthium* was first described, Desvaux (1831) noted that it was unlike any other known genus. Nees ab Esenbeck (1841) placed it in the tribe Phleoideae whereas other members of *Pentaschistis* were placed in the tribe Aveneae, in various genera (*Danthonia*, *Eriachne*, and *Triraphis*). Similarly, whereas the other members of the *Pentaschistis* clade were included in an expanded *Danthonia* D.C. by Durand and Schinz (1895), *Prionanthium* was included in *Phalaris*. Relationship of *Prionanthium* to the *Danthonia* group was first suggested by Chippindall (1955) and followed by Watson and Dallwitz (1992 onward). Clayton and Renvoize (1986) include *Pentameris*, *Pentaschistis*, and *Prionanthium* in the tribe Arundineae but regard *Prionanthium* as relatively distantly related to *Pentameris* and *Pentaschistis*, based on the short glumes and entire tipped lemmas of *Prionanthium* compared with the long glumes, bilobed lemmas, and geniculate awns of the other two genera. Davidse (1988) pointed to four similarities in the spikelets of *Prionanthium* and *Pentaschistis*: the presence of multicellular glands (later supported by Linder et al. (1990) and Ellis (1989)), the two fertile florets per spikelet, the

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Figure 5. The left column of squares shows taxa as occurring on oligotrophic or eutrophic soils and the right column of squares shows taxa as sclerophyllous or orthophyllous. Unless otherwise illustrated, all nodes in clade X4 were optimized with statistical support as orthophyllous-leaved and nodes outside this clade were optimized as sclerophyllous. Unless shown, all nodes in clade VII were optimized as eutrophic and nodes outside this clade as oligotrophic. Pie charts show node optimization (as proportional likelihoods) of nodes that are not unambiguously optimized, those subtending a change in leaf anatomy type, or other nodes of interest. Dashes are placeholders for pie charts that are not shown. Nodes are marked with an asterisk (upper left side) when optimization is statistically significant. Accessions marked with a plus sign were omitted from the contingent states test (see text). Clade names (I to VII) are informal names referred to in the text. Unless otherwise indicated, all species are *Pentaschistis*.

Table 4. Number of "events" counted as for the contingent states test. Glands can either remain absent (0 to 0) or they can evolve (0 to 1) against a background of orthophyllous (0) or sclerophyllous (1) leaf type. Glands can remain present (1 to 1) or they can be lost (1 to 0), as shown, against a background of orthophyllous (0) or sclerophyllous (1) leaf type.

	Glands			
	0 to 0	0 to 1	1 to 1	1 to 0
Leaf anatomy type				
Orthophyllous (0 to 0 and 1 to 0)	0	0	88	6
Sclerophyllous (1 to 1 and 0 to 1)	23	0	5	4

similarity in paleas, and the rachilla extension above the upper floret. These results, based on morphological data, are corroborated by our molecular data that show that *Prionanthium* is nested within *Pentaschistis*. The generic circumscription within the *Pentaschistis* clade as it currently stands does not reflect cladistic conventions. This, including the taxonomic position of *P. tysonii*, will be dealt with in a subsequent publication.

Erecting an infrageneric classification for *Pentaschistis* with its 70 species is fraught with difficulties. Currently there are two informal classifications available: the system of Linder and Ellis (1990) based on gland type and presence, leaf characteristics, and spikelet size; and that of Ellis and Linder (1992) based on leaf anatomy. A formal infrageneric classification of *Pentaschistis* would be desirable to aid communication in this relatively large group. However, it would be difficult to recognize a reasonable number of monophyletic subgenera or sections. Specifically, our phylogenetic tree contains six clades of six to 20 species, yet there are several single species or species pairs scattered throughout what is a rather pectinate tree. These isolated species and species pairs would necessitate up to seven further subgenera to be recognized. This topology, combined with the lack of obvious structural markers for these subgenera, would make the subsequent use of these subgenera and their identification in the field difficult. It additionally means that species not yet included in the molecular phylogenetic analysis cannot readily be placed. We therefore (regrettably) do not attempt to erect a subgeneric classification.

EVOLUTION OF LEAF ANATOMY TYPE AND THE PRESENCE OF GLANDS

Both sclerophyllous and orthophyllous leaf anatomy types are found in different genera of the Danthonioideae (Ellis and Linder 1992), but *Pentaschistis* is unusual within the tribe as it includes both leaf anatomy types. We show that leaf anatomy type is a fairly labile character within the genus *Pentaschistis*, varying within well-supported clades.

It is most probable that the multicellular glands evolved twice within the *Pentaschistis* clade, which is surprising given the otherwise rarity of these glands in the Poaceae. This multiple gain might be an artifact due to multiple extinctions of glanded species early on in the divergence of the *Pentaschistis* clade. However, we cannot rule this out, the most likely hypothesis is that there are two origins. Alternatively, these gains may be the result of a "latent homology" in which the predisposition for a feature to evolve is a synapomorphy, not the structure itself (Stone and Hall 2004) (e.g., predisposition to nodulation within the nitrogen-fixing clade of Rosids I [Soltis et al. 1995] and probably eyes in animals [Fernald 2004]. It is also possible that *P. clavata* is of hybrid origin, having inherited the glands from an unknown paternal parent and the chloroplast genome from *P. colorata*.

Linder et al. (1990) hypothesized that the ancestral state in the clade was glandless and that linear glands were the first to evolve. From these, elongate sessile glands evolved and from these clavate glands. The sunken glands are proposed to be the most derived form of these gland types. This hypothesis was partially tested and supported but with low taxon sampling (Gilbert 2001). Our data support this general evolutionary pattern of increasing gland complexity within the clade, although glands may be lost from either type.

Glands evolved at, or near, the evolution of orthophyllous leaves. The rarity of these events precluded the use of the test of correlated evolution in Discrete (Pagel 1994), which tests whether the transition rate in one (dependent) character is dependent upon the state of a second (independent) character. For our data, the

Table 5. Number of "events" of the two leaf anatomy types onto or within oligotrophic (0) soils or onto or within eutrophic (1) soils as well as pooled results for each soil type in bold.

		0 to 0	1 to 0	Oligotrophic (total)	1 to 1	0 to 1	Eutrophic (total)
Leaf anatomy type	orthophyllous (0 to 0 and 1 to 0)	31	7	**38**	35	2	**37**
	sclerophyllous (1 to 1 and 0 to 1)	44	0	**44**	0	2	**2**

estimates of rate parameters were unrealistically high for both transitions that lead to a gain of glands (data not shown). Pagel (1994) suggested that for such values it is not possible to distinguish between different parameter values. This is probably caused by the rare evolution of glands. Consequently, we used ancestral character state reconstruction as the basis for an alternative approach to test these hypotheses.

Gained only twice, the multicellular glands have been lost at least 12 times, a pattern resembling that found in other complex structures. In the *Pentaschistis* clade some of the species are glandless because they are outside the clade in which glands evolved (the *Pentameris* clade [I], the *P. elegans* clade [II], *P. andringitrensis*, *P. basutorum*, and *P. juncifolia*), but this does not explain all glandless taxa. Although glands have been lost against a background of both leaf types, if the opportunities for gland loss are taken into account, lineages with sclerophyllous taxa tend to lose glands much more often compared to orthophyllous-leaved lineages.

It is also interesting to consider the number of diversification (= speciation minus extinction) events associated with different character combinations. Eglandular lineages with sclerophyllous leaves or glandular lineages with orthophyllous leaves represent the most "stable" character combinations, and are associated with the most diversification events. In contrast, lineages with sclerophyllous leaves and glands are associated with little lineage diversification, and lineages with neither sclerophyllous leaves nor glands arise several times (see Fig. 3), but none of these diversify. Whether this is due to a lower speciation rate or higher extinction rate is not clear, but these lineages may be "evolutionary dead-ends."

The loss of a structure may reflect selective forces different from those with which it originated (Gould 1997). However, we propose a current function of the complex multicellular glands in the *Pentaschistis* clade by considering both character state correlations and the differences in the number of diversification events associated with different character state combinations. Specifically we hypothesize that sclerophyllous leaves and glands each act as a defense system against herbivory. Sclerophylly (Grubb 1986; Turner 1994), lignin (Moore and Jung 2001), and fiber content (Coley 1983; Grubb 1986; Turner 1994; Moore and Jung 2001) in leaves play a primary role in deterring herbivores. Likewise secretion from the glands of the *Pentaschistis* clade has a strong deterrent effect against insects in a choice-chamber experiment (H. P. Linder, pers. obs.), although the active component of the exudates is unknown. The importance of such defense systems in this group is illustrated by the lineages that lose both the glands and the sclerophyllous leaf type that is lose of both defense systems. Although these multiple gland losses are puzzling to explain, the absence of diversification of these lineages supports our hypothesis. This compares with losses of glands occurring alongside a transformation from orthophyllous to sclerophyllous leaf type. These represent an effective swap in the defense system and many of these lineages do diversify, retaining the sclerophyllous leaves as the defense system.

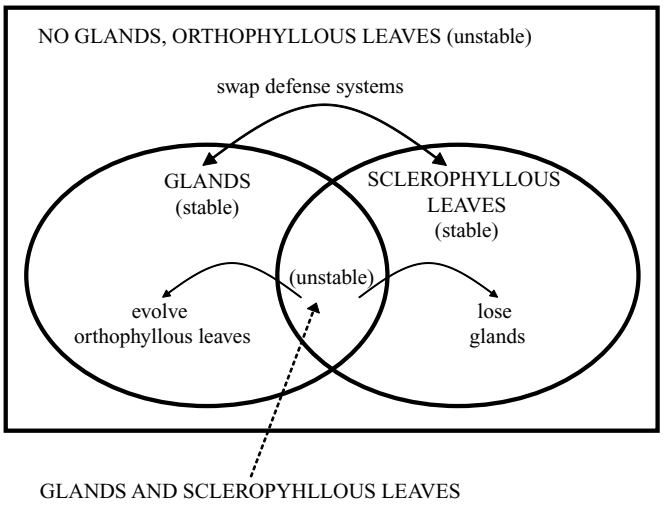

Figure 6. Model of defense system hypothesis for the *Pentaschistis* clade.

Given that there is a cost to producing and/or maintaining sclerophyllous leaves (Sobrado 1991) and glands, a cost/benefit scenario explains these observations and can be applied to the herbivory hypothesis outlined above (see Fig. 6). Having exclusively either defense system is the most "stable" state, as the benefit in terms of defense against herbivory outweighs the cost. Having no defense system (orthophyllous leaves and no glands) is disadvantageous and lineages fail to diversify, (re)evolve either the glands or sclerophyllous leaves, or presumably go extinct. Conversely, if a plant has both systems redundancy means the benefit of the second system does not outweigh its cost, and this "unstable" situation leads to the loss of one of the defense mechanisms.

In considering the diversification of lineages, we combine several unknown factors, including the rates of diversification and extinction, as well as character state change. Although this means that we cannot estimate the rates of character state changes alone, we have the advantage of viewing the outcome of evolution. Specifically, we record the overall effect of the selective disadvantage/advantage of the different character combinations.

What remains to be understood is the "drive" for such a change between the two alternative defense systems. To address this we tested the occurrence of taxa with different leaf types on soils with varying nutrient levels (eutrophic vs. oligotrophic). The CFR is well known for its mosaic of bedrock types, which give rise to both oligotrophic (notably sandstone) and eutrophic soils. The bedrock variety on which the *Pentaschistis* clade grows is further varied due to its distribution on the more eutrophic basalts of the Drakensberg and tropical East African volcanoes. The lability

of soil nutrient level across the phylogeny and the oversimplified partitioning of this continuous character into a binary character was probably the cause of the reduced statistical confidence in the node optimization.

Soil type does not explain the swap in defense systems from glands to sclerophyllous leaves, as lineages with orthophyllous leaf type (with glands) are just as likely to move onto oligotrophic soils as their sclerophyllous (glandless) relatives. There is, however, a significant bias between the two characters as diversification of sclerophyllous lineages is restricted to oligotrophic soils, whereas lineages of orthophyllous type evolve onto and diversify on both soil types. We suggest that the evolution of glands allowed orthophyllous taxa to persist and to diversify onto these more eutrophic soils, although they later revert to and diversify on oligotrophic soils.

Sclerophyllous leaves are associated with not only low nutrient level soils (especially P and N) (Beadle 1966; Cowling and Campbell 1983; Specht and Rundel 1990), but also water deficit (Schimper 1903; Connor and Doley 1981; Lamont et al. 2002). Across the CFR, where most of the *Pentaschistis* species exist, there is a strong rainfall seasonality gradient. One extreme is in the northwest (including Namaqualand), with its pronounced regime of winter rainfall and long summer drought, through to the eastern part of the CFR, with two rain seasons and no water deficit period (Schulze 1997). The occurrence of sclerophylly in the *Pentaschistis* clade, however, does not correlate with this. There are orthophyllous species found in Namaqualand and drier parts of the CFR, and sclerophyllous species found in the eastern part. Consequently, it seems unlikely that sclerophylly is influenced strongly by water deficit.

Nutrient stress and water deficit, however, are notoriously difficult to tease apart (Cunningham et al. 1999; Fonseca et al. 2000) and may have the same effects on the plant and/or even compound each other (Cunningham et al. 1999). Whether soil nutrient level was the primary factor in the evolution of the orthophyllous leaf type, but it has played an important role in determining the differences in diversification between leaf anatomy types in the *Pentaschistis* clade, enabled by the evolution of glands.

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Appendix. Collection and voucher data for DNA samples.

Taxon	Collection	Collection locality and voucher location	trnLF	trnTL	atpB-rbcL	rpL16	trnD
Merxmuellera rufa (Nees) Conert	T. van der Niet 11	Viljoenshof, Western Cape, South Africa (Z)	DQ913471	DQ913558	DQ913240		
Merxmuellera stricta (Schrad.) Conert	T. van der Niet 15	Uilkraal, Western Cape, South Africa (Z)	DQ913472	DQ913559	DQ913241	DQ913326	DQ913407
Pentameris distichophylla (Lehm.) Nees	A. Verboom 226	Hexrivier mountains, Western Cape, South Africa (BOL)	DQ913473	DQ913560	DQ913242	DQ913327	DQ913408
Pentameris glacialis N. P. Barker	H. P. Linder 5498	Great Swartberg, Western Cape, South Africa (BOL)	DQ913474	DQ913561	DQ913243		
Pentameris hirtiglumis N. P. Barker	H. P. Linder 7789	Hottentots Holland Nature Reserve, Western Cape, South Africa (Z)	DQ913475		DQ913244	DQ913328	DQ913409
Pentameris longiglumis (Nees) Stapf	C. Galley 536	Rockview Dam, Western Cape, South Africa (Z)	DQ913476	DQ913562	DQ913245	DQ913329	DQ913410
Pentameris macrocalycina (Steud.) Schweick.	A. Verboom 203	Cape Peninsula, Western Cape, South Africa (BOL)	DQ913477	DQ913563	DQ913246	DQ913330	DQ913411
Pentameris oreophila N. P. Barker	H. P. Linder 7802	Fonteintjiesberg, Western Cape, South Africa (Z)	DQ913478	DQ913564	DQ913247	DQ913331	DQ913412
Pentameris swartbergensis N. P. Barker	H. P. Linder 5490	Klein Swartberg, Western Cape, South Africa (BOL)	DQ913479	DQ913565	DQ913248		DQ913413
Pentameris thuarii Beauv.	H. P. Linder 5456	Montagu Pass, Western Cape, South Africa (BOL)	DQ913480	DQ913566	DQ913249	DQ913332	DQ913414
Pentaschistis acinosa Stapf	T. van der Niet 1	Landdroskop, Western Cape, South Africa (Z)	DQ913481	DQ913567	DQ913250	DQ913333	DQ913415
Pentaschistis airoides (Nees) Stapf subsp. *airoides*	H. P. Linder 6971	Kamiesberg, Northern Cape, South Africa (BOL)	DQ913482	DQ913568	DQ913251		DQ913416
Pentaschistis airoides (Nees) Stapf subsp. *jugorum* (Stapf) H. P. Linder	C. Galley 81	Naudes Nek, Eastern Cape, South Africa (Z)	DQ913483	DQ913569	DQ913252	DQ913334	DQ913417
Pentaschistis alticola H. P. Linder	C. Galley 377	Ceres, Western Cape, South Africa (Z)		DQ913570	DQ913253	DQ913335	DQ913418
Pentaschistis ampla (Nees) McClean	A. Verboom 197	Paarl District, Western Cape, South Africa (BOL)	DQ913484	DQ913571	DQ913254	DQ913336	
Pentaschistis andringitrensis A.Camus	C. Galley 595	Massif d'Andringitra, Madagascar (Z)	DQ913485	DQ913572	DQ913255	DQ913337	DQ913419
Pentaschistis argentea Stapf	A. Verboom 254	Cape Peninsula, Western Cape, South Africa (BOL)	DQ913486	DQ913573	DQ913256	DQ913338	
Pentaschistis aristidoides (Thunb.) Stapf	T. van der Niet 37	Steenbergplateau, Western Cape, South Africa (Z)	DQ913487	DQ913574	DQ913257	DQ913339	
Pentaschistis aristifolia Schweick.	C. Galley 388	Clanwilliam, Western Cape, South Africa (Z)	DQ913488	DQ913575	DQ913258	DQ913340	DQ913420

Continued.

Appendix. Continued.

Taxon	Collection	Collection locality and voucher location	trnLF	trnTL	atpB-rbcL	rpL16	trnD
Pentaschistis aspera (Thunb.) Stapf	A. Verboom 199	Cape Peninsula, Western Cape, South Africa (BOL)	DQ913489	DQ913576	DQ913259	DQ913341	
Pentaschistis aurea (Steud.) McClean subsp. aurea	H. P. Linder 6813	Katberg Pass, Western Cape, South Africa (BOL)	DQ913490	DQ913577	DQ913260		DQ913421
Pentaschistis aurea (Steud.) McClean subsp. *pilosogluma* (McClean) H. P. Linder	C. Galley 47	Monk's Cowl, Kwazulu-Natal, South Africa (Z)	DQ913491	DQ913578	DQ913261	DQ913342	DQ913422
Pentaschistis barbata (Nees) H. P. Linder	A. Verboom 219	Holbaai, Western Cape, South Africa (BOL)	DQ913492		DQ913262	DQ913343	
Pentaschistis basutorum Stapf	C. Galley 44	Golden Gate National Park, Free State, South Africa (Z)	DQ913493	DQ913579	DQ913263	DQ913344	DQ913423
Pentaschistis borussica (K. Schum.) Pilg.	H. P. Linder 7661	Oromiya, Ethiopia (ETH)	DQ913494	DQ913580	DQ913264	DQ913345	DQ913424
Pentaschistis borussica (K. Schum.) Pilg.	C. Galley 230	Mount Elgon, Kenya (Z)	DQ913495	DQ913581	DQ913265	DQ913346	DQ913425
Pentaschistis borussica (K. Schum.) Pilg.	M. Namanganda 1353	Mt. Elgon, Uganda (Z)	DQ913496	DQ913582	DQ913266	DQ913347	DQ913426
Pentaschistis calcicola H. P. Linder var. *calcicola*	C. Galley 338	Bredasdorp, Western Cape, South Africa (Z)	DQ913497	DQ913583	DQ913267	DQ913348	DQ913427
Pentaschistis calcicola H. P. Linder var. *hirsuta* H. P. Linder	C. Galley 339	Bredasdorp, Western Cape, South Africa (Z)	DQ913498	DQ913584	DQ913268	DQ913349	DQ913428
Pentaschistis capensis (Nees) Stapf	H. P. Linder 6825	Bainskloof - Baviaanskloof, Western Cape, South Africa (BOL)	DQ913499	DQ913585	DQ913269	DQ913350	
Pentaschistis capillaris (Thunb.) McClean	C. Galley 322	Vredendal, Western Cape, South Africa (Z)	DQ913500	DQ913586	DQ913270	DQ913351	DQ913429
Pentaschistis caulescens H. P. Linder	C. Galley 376	Ceres, Western Cape, South Africa (Z)	DQ913501	DQ913587	DQ913271	DQ913352	DQ913430
Pentaschistis chippindalliae H. P. Linder	C. Galley 96	Long Tom's Pass, Mpumalanga, South Africa (Z)	DQ913502	DQ913588	DQ913272	DQ913353	DQ913431
Pentaschistis cirrhulosa (Nees) H. P. Linder	C. Galley 548	Stillbaai, Western Cape, South Africa (Z)	DQ913503	DQ913589	DQ913273	DQ913354	
Pentaschistis clavata Galley	C. Galley 567	Ceres, Western Cape, South Africa (Z)	DQ913504	DQ913590	DQ913274	DQ913355	DQ913432
Pentaschistis colorata (Steud.) Stapf	A. Verboom 213	Cape Peninsula, Western Cape, South Africa (BOL)	DQ913505	DQ913591	DQ913275	DQ913356	DQ913433
Pentaschistis colorata (Steud.) Stapf	C. Galley 343	Swellendam, Western Cape, South Africa (Z)	DQ913506	DQ913592	DQ913276		

Continued.

Appendix. Continued.

Taxon	Collection	Collection locality and voucher location	trnLF	trnTL	atpB-rbcL	rpL16	trnD
Pentaschistis colorata (Steud.) Stapf	C. Galley 538	Gysmanshoek Pass, Western Cape, South Africa (Z)	DQ913507		DQ913277	DQ913357	DQ913434
Pentaschistis curvifolia (Schrad.) Stapf	T. van der Niet 53	Galgeberg, Western Cape, South Africa (Z)	DQ913508	DQ913593	DQ913278	DQ913358	DQ913435
Pentaschistis densifolia (Nees) Stapf	A. Verboom 225	Hexrivier mountains, Western Cape, South Africa (BOL)	DQ913509	DQ913594	DQ913279	DQ913359	DQ913436
**Pentaschistis ecklonii* (Nees) McClean	H. P. Linder 6136	Malmesbury, Western Cape, South Africa (BOL)			DQ913280		
Pentaschistis elegans (Nees) Stapf	C. Galley 336	Bredasdorp, Western Cape, South Africa (Z)	DQ913510	DQ913595	DQ913281	DQ913360	DQ913437
Pentaschistis eriostoma (Nees) Stapf	H. P. Linder P6	Western Cape, South Africa (Z)	DQ913511	DQ913596	DQ913282	DQ913361	
Pentaschistis exserta H. P. Linder	C. Galley 51	Drakensberg, Kwazulu-Natal, South Africa (Z)	DQ913512	DQ913597	DQ913283	DQ913362	DQ913438
Pentaschistis galpinii (Stapf) McClean	C. Galley 42	Mount aux Sources, Kwazulu-Natal, South Africa (Z)	DQ913513	DQ913598	DQ913284	DQ913363	
Pentaschistis glandulosa (Schrad.) H. P. Linder	H. P. Linder 6814	Katberg Pass, Eastern Cape, South Africa (BOL)	DQ913514	DQ913599	DQ913285	DQ913364	DQ913439
Pentaschistis heptamera (Nees) Stapf	C. Galley 356	Uitenhague, Eastern Cape, South Africa (Z)	DQ913515	DQ913600	DQ913286	DQ913365	
Pentaschistis horrida Galley	T. van der Niet 20	Seweweekspoort, Western Cape, South Africa (Z)	DQ913516	DQ913601	DQ913287	DQ913366	DQ913440
**Pentaschistis insularis* (Hemsl.) H. P. Linder	M. Lebouvier	BMG Junction, Amsterdam Island (private)	DQ913517				
Pentaschistis juncifolia Stapf	C. Galley 341	Swellendam, Western Cape, South Africa (Z)	DQ913518	DQ913602	DQ913288	DQ913367	DQ913441
Pentaschistis lima (Nees) Stapf	H. P. Linder 6972	Kamiesberg, Northern Cape, South Africa (BOL)	DQ913519	DQ913603	DQ913289	DQ913368	DQ913442
**Pentaschistis longipes* Stapf	H. P. Linder 5018	Humansdorp, Western Cape, South Africa (BOL)			DQ913290		
Pentaschistis malouinensis (Steud.) Clayton	A. Verboom 218	Cape Peninsula, Western Cape, South Africa (BOL)	DQ913520	DQ913604	DQ913291	DQ913369	
Pentaschistis microphylla (Nees) McClean	C. Galley 76	Rhodes, Eastern Cape, South Africa (Z)	DQ913521	DQ913605	DQ913292	DQ913370	DQ913443
Pentaschistis montana H. P. Linder	C. Galley 574	Ceres, Western Cape, South Africa (Z)	DQ913522	DQ913606	DQ913293	DQ913371	
Pentaschistis natalensis (Stapf)	C. Galley 592	Ankaratra, Madagascar (Z)	DQ913523			DQ913372	
Pentaschistis natalensis (Stapf)	C. Galley 69	Mlunje Plateau, Malawi (Z)	DQ913524	DQ913607	DQ913294	DQ913373	DQ913444

Continued.

Appendix. Continued.

Taxon	Collection	Collection locality and voucher location	trnLF	trnTL	atpB-rbcL	rpL16	trnD
Pentaschistis natalensis (Stapf)	C. Galley 95	Long Tom's Pass, Mpumalanga, South Africa (Z)	DQ913525	DQ913608	DQ913295	DQ913374	DQ913445
Pentaschistis oreodoxa (Schweick.)	C. Galley 32	Mount aux Sources, Kwazulu-Natal, South Africa (Z)	DQ913526	DQ913609	DQ913296	DQ913375	DQ913446
Pentaschistis pallescens (Schrad.) Stapf	A. Verboom 216	Cape Peninsula, Western Cape, South Africa (BOL)	DQ913527	DQ913610	DQ913297	DQ913376	DQ913447
Pentaschistis pallida (Thunb.) H. P. Linder form B	T. van der Niet 32	Kogelberg, Western Cape, South Africa (Z)	DQ913528	DQ913611	DQ913298	DQ913377	
Pentaschistis pallida (Thunb.) H. P. Linder form C	C. Galley 547	Stillbaai, Western Cape, South Africa (Z)	DQ913529	DQ913612		DQ913378	
Pentaschistis papillosa (Steud.) H. P. Linder	A. Verboom 209	Silvermine Nature Reserve, Western Cape, South Africa (BOL)	DQ913530	DQ913613		DQ913379	
Pentaschistis patula (Nees) Stapf	C. Galley 317	Clanwilliam, Western Cape, South Africa (Z)	DQ913531	DQ913614	DQ913299	DQ913380	DQ913448
Pentaschistis pictigluma (Steud.) Pilger var. *gracilis* (S. M. Phillips) S. M. Phillips	H. P. Linder 7676	Oromiya, Ethiopia (ETH)	DQ913532	DQ913615	DQ913300	DQ913381	DQ913449
Pentaschistis pictigluma (Steud.) Pilger var. *gracilis* (S. M. Phillips) S. M. Phillips	M. Namanganda 1358	Mt. Elgon, Uganda (Z)	DQ913533		DQ913301	DQ913382	DQ913450
Pentaschistis pictigluma (Steud.) Pilger var. *mannii* S.M. Phillips	C. Galley 267	Mount Kenya, Kenya (Z)	DQ913534	DQ913616	DQ913302	DQ913383	DQ913451
Pentaschistis pictigluma (Steud.) Pilger var. *minor* S. M. Phillips	H. P. Linder 7671	Bale Mountains, Ethiopia (ETH)	DQ913535	DQ913617	DQ913303	DQ913384	
Pentaschistis pictigluma (Steud.) Pilger var. *minor* S. M. Phillips	C. Galley 270	Mount Kenya, Kenya (Z)	DQ913536	DQ913618	DQ913304	DQ913385	DQ913452
Pentaschistis pictigluma (Steud.) Pilger var. *pictigluma* S. M. Phillips	H. P. Linder 7670	Bale Mountains, Ethiopia (ETH)	DQ913537	DQ913619	DQ913305	DQ913386	DQ913453
Pentaschistis pseudopallescens H. P. Linder	C. Galley 379	Hexrivier mountains, Western Cape, South Africa (Z)	DQ913538	DQ913620	DQ913306	DQ913387	DQ913454
Pentaschistis pungens H. P. Linder	C. Galley 333	Clanwilliam, Western Cape, South Africa (Z)	DQ913539	DQ913621	DQ913307	DQ913388	DQ913455
Pentaschistis pusilla (Nees) H. P. Linder	A. Verboom 206	Cape Peninsula, Western Cape, South Africa (BOL)	DQ913540	DQ913622	DQ913308	DQ913389	DQ913456
Pentaschistis pyrophila H. P. Linder	A. Verboom 229	Hexrivier mountains, Western Cape, South Africa (BOL)	DQ913541	DQ913623	DQ913309	DQ913390	

Continued.

Appendix. Continued.

Taxon	Collection	Collection locality and voucher location	trnLF	trnTL	atpB-rbcL	rpL16	trnD
Pentaschistis reflexa H. P. Linder	C. Galley 324	Clanwilliam, Western Cape, South Africa (Z)	DQ913542	DQ913624	DQ913310	DQ913391	DQ913457
Pentaschistis rigidissima Pilger ex H. P. Linder	A. Verboom 227	Hexrivier mountains, Western Cape, South Africa (BOL)	DQ913543	DQ913625	DQ913311	DQ913392	DQ913458
Pentaschistis rosea H. P. Linder subsp. *purpurascens* H. P. Linder	C. Galley 378	Hexrivier mountains, Western Cape, South Africa (Z)	DQ913544	DQ913626	DQ913312	DQ913393	DQ913459
Pentaschistis rupestris (Nees) Stapf	A. Verboom 251	Hexrivier mountains, Western Cape, South Africa (BOL)	DQ913545	DQ913627	DQ913313	DQ913394	DQ913460
Pentaschistis scandens H. P. Linder	C. Galley 334	Bredasdorp, Western Cape, South Africa (Z)	DQ913546	DQ913628	DQ913314	DQ913395	
Pentaschistis setifolia (Thunb.) McClean	C. Galley 45	Golden Gate National Park, Free State, South Africa (Z)	DQ913547	DQ913629	DQ913315	DQ913396	DQ913461
Pentaschistis tomentella Stapf	C. Galley 318	Calvinia, Northern Cape, South Africa (Z)	DQ913548	DQ913630	DQ913316	DQ913397	DQ913462
Pentaschistis tortuosa (Trin.) Stapf	A. Verboom 250	Hexrivier mountains, Western Cape, South Africa (BOL)	DQ913549	DQ913631	DQ913317	DQ913398	DQ913463
Pentaschistis trifida. Galley	C. Galley 577	Ceres, Western Cape, South Africa (Z)	DQ913550	DQ913632	DQ913318	DQ913399	DQ913464
Pentaschistis triseta (Thunb.) Stapf	H. P. Linder 6962	Romansrivier, Western Cape, South Africa (BOL)	DQ913551	DQ913633	DQ913319	DQ913400	DQ913465
Pentaschistis tysonii Stapf	H. P. Linder 6812	Katberg Pass, Western Cape, South Africa (BOL)	DQ913552	DQ913634	DQ913320	DQ913401	DQ913466
Pentaschistis velutina H. P. Linder	C. Galley 389	Clanwilliam, Western Cape, South Africa (Z)	DQ913553	DQ913635	DQ913321	DQ913402	DQ913467
Pentaschistis veneta H. P. Linder	C. Galley 576	Ceres, Western Cape, South Africa (Z)	DQ913554	DQ913636	DQ913322	DQ913403	DQ913468
Pentaschistis viscidula (Nees) Stapf	H. P. Linder 7787	Hottentots Holland Nature Reserve, Western Cape, South Africa (Z)	DQ913555	DQ913637	DQ913323	DQ913404	
Prionanthium dentatum (L. f.) Henrard	H. P. Linder 5430	Nieuwoudtville, Western Cape, South Africa (BOL)	DQ913556	DQ913638	DQ913324	DQ913405	DQ913469
Prionanthium ecklonii (Nees) Stapf	H. P. Linder 5402	Clanwilliam, Western Cape, South Africa (BOL)	DQ913557	DQ913639	DQ913325	DQ913406	DQ913470

* Material obtained from herbarium specimen. Codes are Genbank accession codes.