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Molecular phylogeny of *Phebalium* (Rutaceae: Boronieae) and related genera based on the nrDNA regions ITS 1 + 2

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Abstract. Parsimony analyses of the internal transcribed spacer regions of nuclear ribosomal DNA (ITS 1 & ITS 2) for 38 taxa sampled from the *Phebalium* group (Rutaceae: Boronieae) and two outgroups confirm that, with the exception of *Phebalium* sensu stricto *and Rhadinothamnus*, six of the currently recognised genera within the group are monophyletic. The data indicate that *Phebalium* s. str. is paraphyletic with respect to *Microcybe*, and *Rhadinothamnus* is paraphyletic with respect to *Chorilaena*. *Rhadinothamnus* and *Chorilaena* together are the sister group to *Nematolepis*. *Drummondita*, included as an outgroup taxon, clustered within the ingroup as sister to *Muiriantha* and related to *Asterolasia*.

The phylogeny suggests that the evolution of major clades within a number of these genera (e.g. *Phebalium*) relates to vicariance events between eastern and south-western Australia. *Leionema* is an eastern genus, with the most basal taxon being the morphologically distinct *Leionema ellipticum* from northern Queensland. *Leionema* also includes one species from New Zealand, but this species (as with some others) proved difficult to sequence and its phylogenetic position remains unknown. Taxonomic changes at the generic level are recommended.

Key words: Rutaceae, *Phebalium*, *Leionema*, *Rhadinothamnus*, *Nematolepis*, *Chorilaena*, *Asterolasia*, *Microcybe*, *Muiriantha*, *Drummondita*, ITS, phylogeny, biogeography.

Introduction

The *Phebalium* group *sensu* Wilson (1998) (Rutaceae: Boronieae, Table 1) comprises nine genera and c. 97 species of woody shrubs, which, with the exception of *Leionema nudum* (Hook.) Paul G. Wilson from the North Island of New Zealand, are confined to temperate south-western and eastern Australia (Fig. 1). Australian taxa occur in a range of plant communities, including forests, alpine and coastal heathlands and shrublands, semi-arid heathlands and mallee communities. Most taxa are restricted to specific regions and many are highly localised endemics. Species are cultivated for their utility in horticulture, although many with recognised potential have yet to be utilised.

Members of the *Phebalium* group have simple, terete to more or less flat, alternate leaves that lack stipules. The fleshy or chartaceous leaves are often glandular-verrucose, and many species have an indumentum of simple (e.g. some species of *Leionema* (F. Muell.) Paul G. Wilson), stellate (e.g.

Table 1. Genera in the *Phebalium* group *sensu* Wilson (1998) and the Australian States in which they occur, with one species from New Zealand

Genus	No. species	Distribution
Asterolasia F.Muell.	16	Q, N, V, S, W
Chorilaena Endl.	1	W
Diplolaena R.Br.	15	W
Leionema (F.Muell.) Paul G. Wilson	23	Q, N, V, T, S, NZ
Microcybe Turcz.	3	V, S, W
Muiriantha C.A. Gardner	1	W
Nematolepis Turcz.	7	N, V, T, W
Phebalium Vent.	28	Q, N, V, T, S, W
Rhadinothamnus Paul G. Wilson	3	W

Q = Queensland, N = New South Wales, V = Victoria, T = Tasmania, S = South Australia, W = Western Australia, NZ = New Zealand

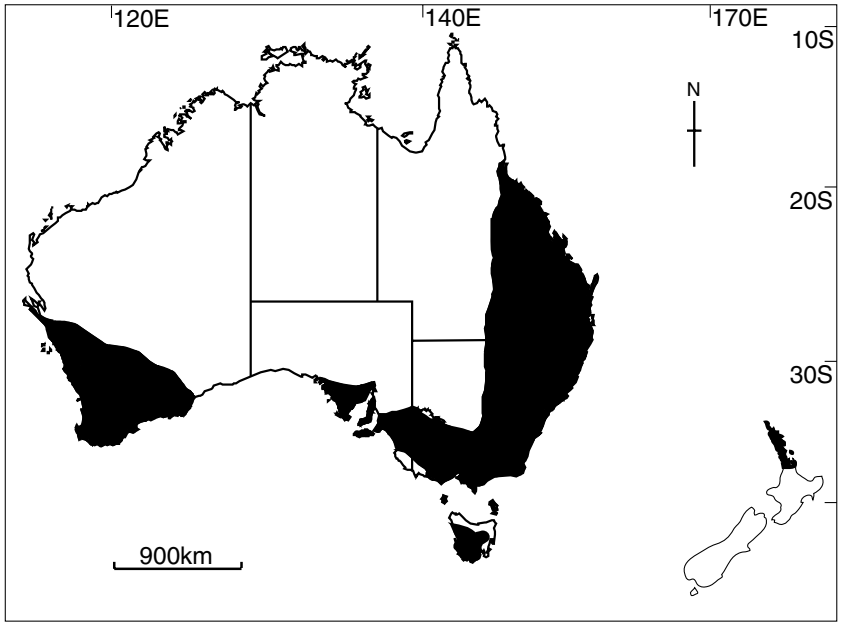

Fig. 1. Distribution of the *Phebalium* group *sensu* Wilson (1998) in Australia. One species of *Leionema* is endemic to the north-west of the North Island of New Zealand

Asterolasia F. Muell.) or peltate trichomes (*Rhadinothamnus* Paul G. Wilson, *Nematolepis* Turcz. and *Phebalium* Vent.), while others are glabrous (e.g. most species of *Leionema*). As with other members of the Rutaceae, many of the organs are covered with pellucid oil glands.

Flowers are usually pentamerous, although up to eight petals have been observed in *Phebalium nottii* (F. Muell.) Maiden et Betche. The 10 stamens are free and spread at anthesis, a character that is considered to be a synapomorphy for the *Phebalium* group *sensu* Armstrong (1991, unpublished data).

Currently there is no published phylogenetic analysis to test the monophyly and relationships of taxa within the *Phebalium* group. Thus the aim of this first paper was to use sequence data from the internal transcribed spacer regions of nuclear ribosomal DNA (ITS 1 & ITS 2) for a phylogenetic analysis of exemplar species

from the genera of the *Phebalium* group *sensu* Wilson (1998) and to use the resulting phylogenetic tree as the basis for a biogeographic analysis. Subsequent papers are aimed at analysis of morphological characters in comparison with molecular results for formal taxonomic revision where needed.

Taxonomic history of the *Phebalium* group

Phebalium was first described by Ventenat (1805) based on a specimen of *P. squamulosum* Vent. from New South Wales. He incorrectly described the five carpellary ovary as being united, and placed the genus in the Myrtaceae, considering it to be closely related to *Baeckea* L. and *Leptospermum*. J.R.Forst. & G.Forst. Smith (1814) later recognised that Ventenat's description of *Phebalium* applied to specimens he had received from Australia that did not have united carpels, and transferred the genus to the Rutaceae.

In 1824 A. P. de Candolle recognised two species (*P. squamulosum* and *P. anceps* DC.). The following year, Jussieu (1825) divided the genus into three sections to which he did not give names. The first section contained two species now included in the genus *Asterolasia*. Endlicher (1840) described section *Eriostemoides* Endl. followed by the description of section *Lepidota* Rchb. (Reichenbach 1841) to accommodate those species with lepidote hairs. In 1859 Mueller transferred the genus to *Eriostemon* Sm. and then later (Mueller 1862) divided it into six sections, three of which included taxa previously included in *Phebalium*. *Eriostemon* section *Phebalium* included species with lepidote hairs, sect. *Leionema* F.Muell. included species possessing leaves that were mostly glabrous and always without lepidote hairs, and section *Chorilaenopsis* F.Muell. included a single species, *Eriostemon phylicoides* F.Muell. (now *Leionema diosmeum* (Juss.) Paul G.Wilson), which has scabrous-hairy leaves.

In 1863 Bentham reinstated *Phebalium* as a genus, but limited it to sections *Euphebalium* Benth. (Mueller's sect. *Phebalium*) and *Leionema* (including Mueller's sect. *Chorilaenopsis*). Although Baillon (1886) transferred *Phebalium* to *Eriostemon* and then later in the same work to *Crowea* Sm., this has not been recognised by other botanists, and Bentham's treatment was subsequently followed by Engler (1896, 1931).

Smith-White (1954), when discussing the taxonomic implications of chromosome numbers in the Boroniae, stated that "*Phebalium* (x = 16) is clearly distinct" from other genera in the tribe. The cytological data for the Boroniae presented by Smith-White do not support Mueller's (1859) treatment of the group, but rather, support the treatments of Bentham (1863) and later Engler (1896, 1931).

Wilson (1970) revised *Phebalium* and recognised four sections: *Phebalium*, *Eriostemoides*, *Gonioclados* Paul G. Wilson and *Leionema*. He commented on the relationships of these sections with other genera (see Wilson 1970, 1971) in the *Phebalium* group and considered that some of the sections of *Phebalium* had greater affinity with these other genera than with other sections within the genus. Recognising *Phebalium* as polyphyletic, Wilson (1998) transferred section *Gonioclados* to *Rhadinothamnus* (previously monotypic), section *Eriostemoides* to *Nematolepis* (previously monotypic), raised section *Leionema* to genus level, and suggested a close relationship between section *Phebalium* and *Microcybe* Turcz. However, the relationships of these taxa to one another and to *Asterolasia*, *Diplolaena* R.Br., *Muiriantha* C.A. Gardner and *Chorilaena* Endl. were unclear. Wilson (1998) also discussed the relationships of the north Queensland *Leionema ellipticum*, considering it to be unusual within *Leionema* in having bluntly mucronulate anthers and a deeply grooved gynophore, and suggesting that it may warrant generic status.

Materials and methods

Plant material. Thirty-six taxa in the *Phebalium* group *sensu* Wilson (1998) were sampled (Table 2): nine species of *Phebalium* (32% of species), two species of *Microcybe* (66%), seven species of

Table 2. Collection localities and voucher numbers for taxa sequenced. Vouchers will be lodged at MEL with duplicates distributed to various Australian Herbaria. Abbreviations are; BJM = Bryan John Mole, MFD = Marco Fillipo Duretto

Species	Voucher	Location	Genbank Accession
Asterolasia asteriscophora (F.Muell.) Druce subsp. *asteriscophora*	BJM 224	Gladysdale, Victoria	AY631937
A. muricata J.M.Black	MFD 1366	Kangaroo Island, South Australia	AY631936
A. nivea (Paul G.Wilson) Paul G.Wilson	BJM 364	New Norcia, Western Australia	AY631939
A. pallida Benth. subsp. *pallida*	BJM 489	Nannup, Western Australia	AY631935
A. pallida subsp. *hyalina* Paul G.Wilson	BJM 496	Contine Hill, Western Australia	AY631934
A. phebalioides F.Muell.	MFD 1365	Kangaroo Island, South Australia	AY631938
A. squamuligera (Hook.) Benth.	BJM 462	Lake Grace district, Western Australia	AY631940
Chorilaena quercifolia Endl.	BJM 485	b/w Denmark & Walpole, Western Australia	AY631915
Leionema caruthersii (F.Muell.) Paul G.Wilson	BJM 251	Dr George Mtn, New South Wales	AY631917
L. diosmeum (Comm. ex A.Juss.) Paul G.Wilson	BJM 244	Ben Boyd Nat. Park, New South Wales	AY631920
L. ellipticum Paul G.Wilson	P. Forster 25021	Queensland	AY631916
L. equestre (D.A.Cooke) Paul G.Wilson	MFD 1374	Kangaroo Island, South Australia	AY631922
L. lamprophyllum (F.Muell.) Paul G.Wilson	BJM 283	Licola area, Victoria	AY631918
L. phylicifolium (F.Muell.) Paul G.Wilson	BJM 317	Kosciusko Nat. Park, New South Wales	AY631919
L. ralstonii (F.Muell.) Paul G.Wilson	BJM 248	Egan Peaks, New South Wales	AY631921
Microcybe multiflora Turcz.	BJM 232	Bronzewing, Victoria	AY631932
M. pauciflora Turcz.	MFD 1370	Kangaroo Island, South Australia	AY631933
Muiriantha hassellii (F.Muell.) C.A.Gardner	BJM 474	Stirling Range, Western Australia	AY631911
Nematolepis elliptica (Paul G.Wilson) Paul G.Wilson	BJM 266	Big Badja Mtn, New South Wales	AY631906
N. frondosa (N.G.Walsh & Albr.) Paul G.Wilson	BJM 268	Mt Elizabeth, Victoria	AY631907
N. phebalioides Turcz.	BJM 436	Hopetown, Western Australia	AY631910
N. rhytidophylla (Albr. & N.G.Walsh) Paul G.Wilson	BJM 310	Nalbaugh Plateau, New SouthWales	AY631905
N. squamea (Labill.) Paul G.Wilson subsp. *squamea*	BJM 264	Wanderra State Forest, New SouthWales	AY631908
N. squamea subsp. *coriacea* (Paul G.Wilson) Paul G.Wilson	BJM 276	The Watchtower, Victoria	AY631909
Phebalium ambiguum C.A.Gardner	BJM 362	Wubin, Western Australia	AY631931
P. canaliculatum (F.Muell. & Tate) J.H.Willis	BJM 351	N.E. of Wubin, Western Australia	AY631934
P. clavatum C.A.Gardner	BJM 398	Schahill Timber reserve, Western Australia	AY631923
P. elegans Paul G.Wilson	BJM 403	94kms east of Norseman, Western Australia	AY631925

Table 2 (continued)

Species	Voucher	Location	Genbank Accession
P. festivum Paul G.Wilson	BJM 236	Rushworth, Victoria	AY631928
P. filifolium Turcz.	BJM 373	Tammin, Western Australia	AY631930
P. glandulosum Hook.	BJM 235	Goschen, Victoria	AY631926
P. squamulosum Vent. subsp. *ozothamnoides* (F.Muell.) Paul G.Wilson	BJM 269	Anglers Rest, Victoria	AY631927
P. tuberculosum (F.Muell.) Benth.	BJM 375	Merredin, Western Australia	AY631929
Rhadinothamnus anceps (DC.) Paul G.Wilson	BJM 475	Albany, Western Australia	AY631914
R. euphemiae (F.Muell.) Paul G.Wilson	BJM 424	Cape Le Grand Nat. Park, Western Australia	AY631912
R. rudis (Bartl.) Paul G.Wilson	BJM 441	Ravensthorpe, Western Australia	AY631913
Outgroups			
Crowea exalata F.Muell.	BJM 237	Rushworth, Victoria	AY631903
Drummonditta hassellii (F.Muell.) Paul G.Wilson	BJM 361	Wubin, Western Australia	AY631904

Leionema (30%), six species and two subspecies of *Asterolasia* (38%), three species of *Rhadinothamnus* (100%), five species and two subspecies of *Nematolepis* (71%), and the monotypic genera *Muirianta* and *Chorilaena*. *Crowea* and *Drummondita*, tribe Boronieae, were included as two outgroup taxa. *Geleznowia* was also sequenced for use as a third outgroup but sequences were insufficiently clear. Outgroup taxa were chosen based on an unpublished morphological phylogeny of the Boroniae (Armstrong 1991, unpublished data), which placed the *Phebalium* group in a polytomy comprised of *Drummondita*, *Geleznowia* Turcz. and a clade including *Crowea*.

Fresh plant material was generally sampled from the field throughout Australia, although some material was obtained from cultivated plants. Young leaf material, free of disease and other potential contaminants such as dust and insects, was preferentially chosen for sampling. Vouchers for all taxa were lodged at the National Herbarium of Victoria (MEL). Fresh material was either immediately transported or posted overnight to MEL for freezing at −80 °C for later DNA isolation. Leaf material for all species was also dried rapidly using silica gel as a backup sample.

Isolation of DNA. Initially, isolation of genomic DNA from both fresh and dry material was performed using the modified CTAB protocol as in Udovicic et al. (1995). Subsequently, all DNA isolation was carried out using Nucleospin kits (Macherey-Nagel Düren, Germany). QIAGEN DNeasy mini kits were used briefly but did not yield satisfactory DNA. Genomic DNA was purified using Prep-a-Gene (Bio Rad) following the manufacturer's instructions.

Amplification of ITS. The utility and favorable properties of the ITS regions 1 & 2 of nrDNA for plant systematics is well documented by Baldwin et al. (1995) and others. Although this region consists of multiple repeats, paralogy is not considered a problem due to concerted evolution. Amplification proved to be problematic for Rutaceae and required trialing a number of different PCR conditions before satisfactory amplifications were obtained. The methodology was an evolutionary process, but three main amplification techniques were eventually found to work with varying degrees of success.

Initially primers S3 and S4 of Käss and Wink (1997) were used with limited success. During this initial stage, PCR conditions were: 94 °C for 3 min followed by 30 cycles of denaturation, annealing and extension, being 94 °C for 30 sec, 58 °C for 30 sec and 70 °C for 10 sec respectively. This was followed by a further 5 min extension at 72 °C. Each 50 µl reaction contained 5 µl of 10 × PCR buffer, 0.2 mM of each dNTP, 3 mM $MgCl_2$, 10 pmol each of primers S3 and S4, 1.25 Units of Taq polymerase, 2 µl of genomic DNA. DNA that did not respond well to this method was subjected to a second amplification. This was achieved by stab-

bing the appropriate fluorescent band on an agarose gel with a pipette tip and mixing the tip in a 1.5 ml centrifuge tube containing 20 µl of distilled H_2O. The entire 20 µl was then re-amplified using the same PCR conditions as above.

These techniques resulted in amplification of several taxa but many were still problematic. In an attempt to overcome this, a nested PCR protocol (Udovicic and Murphy 2002) was employed. The nested PCR protocol utilises an initial round of amplification using external primers (in this case ITS18 and ITS26 of Käss and Wink 1997). 1 µl of the resultant PCR product was then re-amplified using the internal primers S3 and S4. It was occasionally necessary to use the additional internal primers S1, S2, S5 and S6, also of Käss and Wink (1997), in order to obtain a complete sequence. Each 50 µl reaction in round one contained 5ul of 10 × buffer, 0.2mM of each dNTP,10pmol each of primers ITS18 and ITS26, 1.25 units of HotStarTaq (QIAGEN) and between 20ng and 100ng of genomic DNA. PCR cycling conditions for round one were 95 °C for 15 min followed by 30 cycles of 94 °C for 30 sec, 60 °C for 30 sec and 72 °C for 30 sec. These 30 cycles were followed by 72 °C for 5 min. In round two, each 50 µl reaction contained 5 µl of 10 × buffer, 0.2mM of each dNTP, 20pmol each of primers S3 and S4, 2.5 units of HotStarTaq (QIAGEN) and between 1 µl and 5 µl of PCR product from the first round PCR. The PCR cycling conditions for round two were the same as round one except that the annealing temperature was reduced from 60 °C for 30 sec to 51 °C for 30 sec.

Initially, both the first and second rounds of PCR cycling conditions utilized stringent annealing temperatures. However, for some taxa that still proved difficult, the second round annealing temperature was reduced. Lowering annealing temperature can potentially result in non-specific priming (Palumbi 1996). However, in this case, there was little risk of non-specific priming occurring because the first round PCR was much more stringent (higher annealing temperature), and consequently it was largely only specific priming sites that were available for binding in the second round.

Purification and sequencing of PCR products. PCR products were either purified using agarose gel electrophoresis followed by gel extraction using QIAquick Gel Extraction kits (QIAGEN) following manufacturer's instructions, or by direct column purification using either CONCERT Rapid PCR Purification System (Gibco BRL) or QIAquick Purification kits (QIAGEN) following the respective manufacturer's instructions.

Sequencing reactions were carried out using an ABI Prism Ready Reaction Dye Terminator Cycle sequencing kit (Perkin-Elmer). The purified PCR products were used as a template for direct sequencing with primers S1, S2, S3, S4, S5, S6, ITS18 and ITS 26. Contiguous sequences were assembled and edited where required with Sequencher v3.0 (Gene Codes Corporation). Sequences were aligned manually using SeqPup v0.6 (Don Gilbert Indiana University). A copy of aligned sequences is available from the corresponding author upon request.

Phylogenetic analysis. Aligned sequences were visually inspected for insertion and deletion events (indels), which were coded as binary or multistate characters, and manually entered at the end of the DNA data matrix. Regions coded as indels were excluded as single base characters from the data matrix with the exception of those indel regions that contained parsimony informative base pair substitutions. In such regions gaps were coded as missing data. A region of 20 base pairs close to the S4 priming site, containing bases of uncertain position was also excluded from the analysis. Individual base positions were coded as unordered multistate characters.

Phylogenetic analyses were completed using parsimony methods in the program PAUP* version 4.0b10 (Swofford 1998). A Heuristic parsimony search of 100 random addition replicates with the tree-bisection-reconnection (TBR) branch swapping algorithm in effect and saving all of the most optimal trees (MULTREES in effect), was conducted. A Bootstrap analysis (Felsenstein 1985) was completed to calculate branch supports for the strict consensus tree using 1000 bootstrap replicates, each with 10 replicates of a random addition sequence.

Results

The ITS region. Amplification and sequencing of ITS 1 & 2 region of nrDNA for taxa in the *Phebalium* group proved problematic (see Methods). Although three amplification protocols were used, the protocol that was most

reliable was a nested PCR strategy (Udovicic and Murphy 2002). As a result of the problems associated with DNA amplification and sequencing, there are no representatives for *Diplolaena*, Tasmanian *Leionema* species, Tasmanian *Phebalium* species, or *Leionema nudum*, the sole New Zealand taxon, included in the ITS data set. Genbank accession numbers for taxa sequenced are listed in Table 2.

The boundaries of the ITS 1 & 2 and the 5.8S gene were identified by reference to the published sequences of Käss and Wink (1997).

Table 3. Sequence lengths for each taxon. An asterisk (*) indicates partial sequences

Taxon	Entire sequence	ITS1	ITS2	5.8S
Asterolasia asteriscophora	619	215	236	168
A. muricata	617*	216	233*	168
A. nivea	609*	213	228*	168
A. phebalioides	610*	212*	230*	168
A. pallida subsp. *hyalina*	619	214	237	168
A. pallida subsp. *pallida*	615	211	236	168
A. squamuligera	600*	202*	230	168
Chorilaena quercifolia	616	214	234	168
Leionema carruthersii	612	212	232	168
L. diosmeum	612	211	233	168
L. ellipticum	618*	212*	237	169
L. equestre	596*	196*	232	168
L. lamprophyllum	612	211	233	168
L. phylicifolium	611	211	232	168
L. ralstonii	603*	203*	232	168
Microcybe multiflora	615	212	235	168
M. pauciflora	616	213	235	168
Muiriantha hassellii	606*	212*	226*	168
Nematolepis elliptica	607*	207*	232	168
N. frondosa	613	213	232	168
N. phebalioides	565*	164*	233	168
N. rhytidophylla	614	213	233	168
N. squamea subsp. *squamea*	609*	208*	233	168
N. squamea subsp. *coriacea*	613	213	232	168
Phebalium ambiguum	616	213	235	168
P. clavatum	603*	202*	233	168
P. canaliculatum	589*	202*	218*	169
P. elegans	613	212	233	168
P. festivum	594*	192*	234	168
P. filifolium	600*	209*	223*	168
P. glandulosum	614	212	234	168
P. squamulosum subsp. *ozothamnoides*	615	213	234	168
P. tuberculosum	604*	212	224*	168
Rhadinothamnus anceps	594*	215	211*	168
R. euphemiae	634	229	237	168
R. rudis	611*	215	228*	168
*Crowea exalata**	598*	204*	227*	167
*Drummondita hassellii**	617	213	236	168

Excluding taxa with partial sequences, the length of sequences for ITS regions 1 & 2 and the 5.8S gene inclusive ranged from 611 bp to 634 bp (see Table 3). For the ITS 1 region, sequence lengths ranged from 211 bp to 216 bp, with one exception being *Rhadinothamnus euphemiae* with a length of 229 bp. The length of ITS 2 sequences ranged from 230 bp to 237 bp. The length of the 5.8S gene was 168 bp for all but three accessions. The exceptions were a length of 169 bp for both *Leionema ellipticum* and *Phebalium canaliculatum* and 167 bp for *Crowea exalata*.

Phylogeny. The entire ITS data matrix for the 38 accessions consisted of 749 characters, including 29 insertion/deletion events coded as binary or multistate characters. Of the 749 characters, 391 were invariant base positions, 105 were variable but uninformative autapomorphies, and 230 were informative synapomorphies. Parsimony analysis including indels resulted in 90 equally parsimonious trees (tree length = 915; CI = 0.5475; RI = 0.7534). The strict consensus tree (Fig. 2) is well resolved, with 29 nodes identified in all most-parsimonious trees, 24 nodes of which had bootstrap support greater than 50%. An analysis (not shown) was also conducted excluding indel characters, and the tree topology was the same. The results indicate that the ITS region is useful for inferring relationships at a number of taxonomic levels in the *Phebalium* group.

The strict consensus tree (Fig. 2) shows one of the outgroup taxa, *Drummondita*, placed within the ingroup, although this placement has no bootstrap support. The strict consensus tree also indicates that six of the currently recognised genera in the *Phebalium* group are monophyletic, with five of these supported by bootstrap values of 84% or more. Exceptions are *Rhadinothamnus*, which is paraphyletic with respect to *Chorilaena*, and *Phebalium*, which is paraphyletic with respect to *Microcybe*. *Diplolaena* was not represented in the analysis; although DNA for two species (*D. angustifolia* Hook. and *D. graniticola* Paul G. Wilson) was successfully amplified, the sequences were extremely unclear despite a number of trials. However, *Diplolaena* is morphologically a well-defined genus whose monophyly is not seriously questioned. Based on a combined analysis of *rbc*L and *atp*B sequence data of Rutaceae, Chase et al. (1999) placed *Diplolaena* within a well-supported clade (Bootstrap support 77%), including *Phebalium* s. str., *Chorilaena*, *Philotheca* and *Correa* Andrews.

The strict consensus tree shows two major clades (A & B, Fig. 2), both of which lack bootstrap support. The branch lengths in Fig. 3 (tree 1 of the 90 equally parsimonious trees) provide an indication of the relative divergence of clades, based on the number of base substitutions and/or indels supporting each node. Indel character transformations are mapped onto tree number one of the 90 most parsimonious trees (Fig. 4, Table 4).

Clade A. Clade A (node 1) is comprised of the genera *Leionema, Muiriantha, Drummondita,* and *Asterolasia*. Node 1 is supported by two indel characters (Characters 8 and 15, Fig. 4), although it lacks bootstrap support. Within clade A, all exemplars of *Leionema* form a monophyletic group (Clade C, node 3, bootstrap support 97%), which is supported by a synapomorphy with later reversal and a parallelism (indel characters 2 and 24, Fig. 4). *Leionema ellipticum* from northern Queensland is sister to all other *Leionema* species sampled, with node 7 having strong bootstrap support (100%). *Leionema ellipticum* may warrant recognition at generic or subgeneric (i.e. sectional) level. It is unique within the genus in possessing a grooved gynophore and bluntly mucronulate anthers (see Wilson 1998). Within the remaining *Leionema* species, the South Australian *L. equestre*, which is endemic to Kangaroo Island, is sister to an eastern states clade (node 8, bootstrap support 75%). *Leionema equestre* is unique within the genus in having a repeatedly divaricate stem architecture (see Cooke 1987). The eastern states clade at node 8 is supported by a synapomorphic deletion event (character 1, Fig. 4). *Leionema carruthersii*, *L. lamprophyllum* and *L. ralstonii* form a mono-

phyletic group at node 10 (bootstrap support 72%). *Leionema carruthersii* and *L. lamprophyllum* are sister taxa (node11, bootstrap support 77%), a finding that conflicts with relationships based on morphology. *Leionema carruthersii* and *L. ralstonii*, together with two other species – *L. viridiflorum* and *L. sympetalum* (not sequenced) – have pendant inflorescences, an erect corolla ± parallel to the stamens and stamens twice as long as the

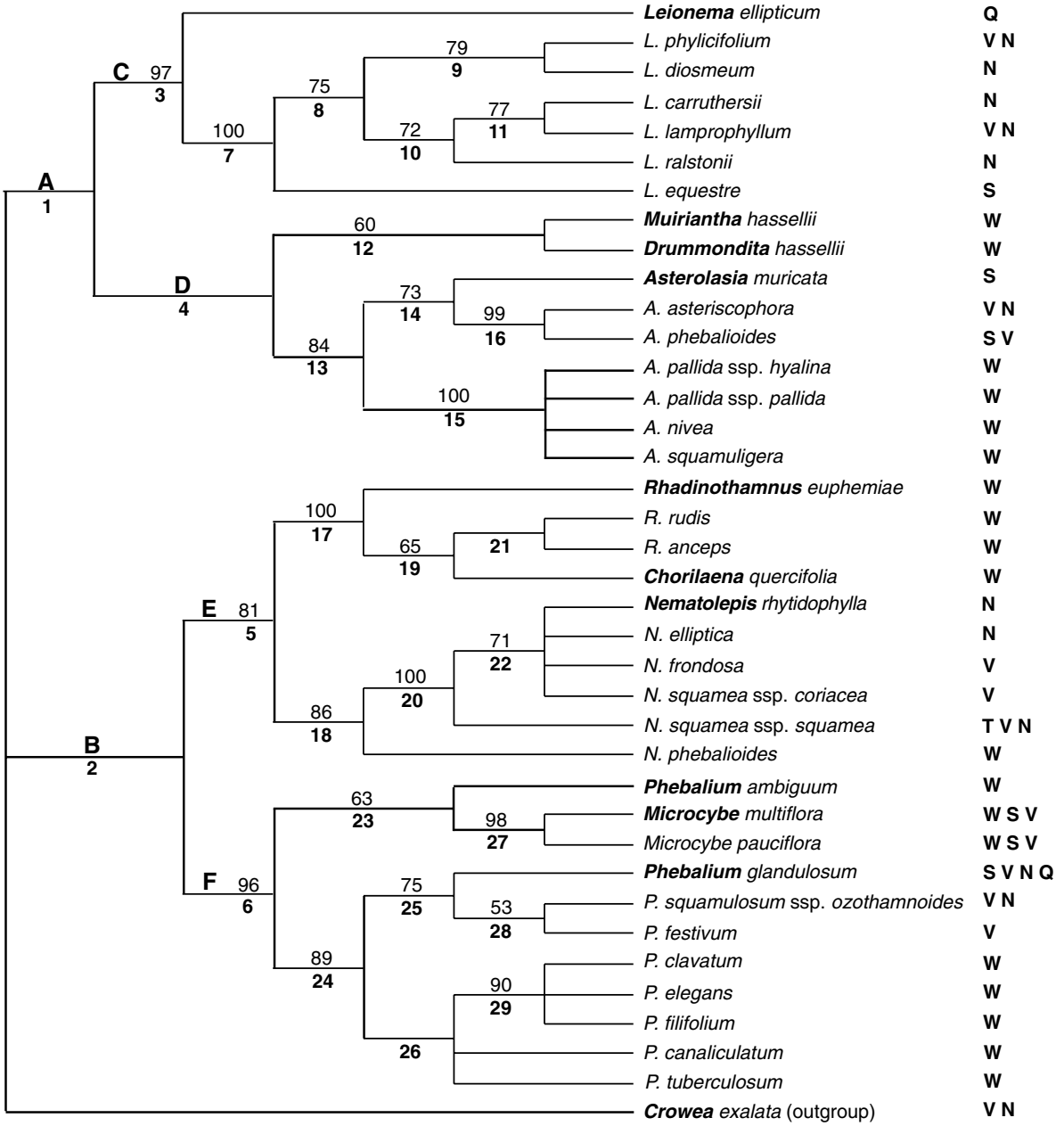

Fig. 2. Strict consensus tree of 90 equally parsimonious trees for the ITS data set. Bootstrap values > 50% are indicated above the branches. Nodes are numbered 1–29 and major clades ***A-F*** are discussed in the text. The distribution of taxa are shown as in the following areas: ***W*** Western Australia, ***S*** South Australia (Kangaroo Island), ***V*** Victoria, ***N*** New South Wales, and ***Q*** Queensland

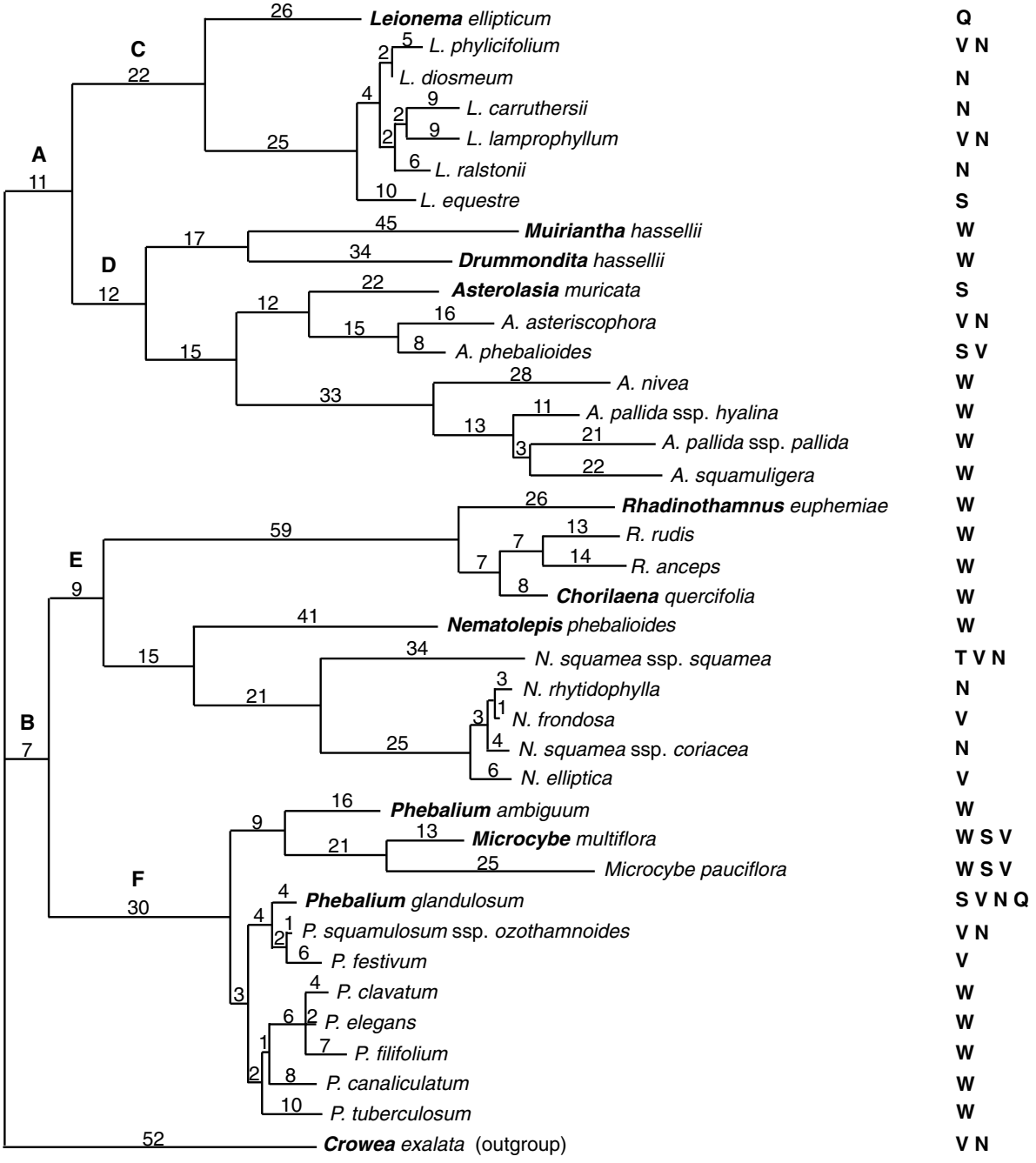

Fig. 3. Tree number one of the 90 most parsimonious trees. Numbers above nodes are the branch lengths, which reflect the degree of divergence in the group based on the number of base substitutions and/or indels supporting each node

corolla. The flowers of *L. lamprophyllum* are arranged in a terminal cluster with the flowers solitary or in clusters of one to three arising from the axils of terminal leaves. The corolla is spreading rather than erect and the stamens are ± equal in length to the corolla. *Leionema phylicifolium* and *L. diosmeum* are sister taxa (node 9, bootstrap support 79%).

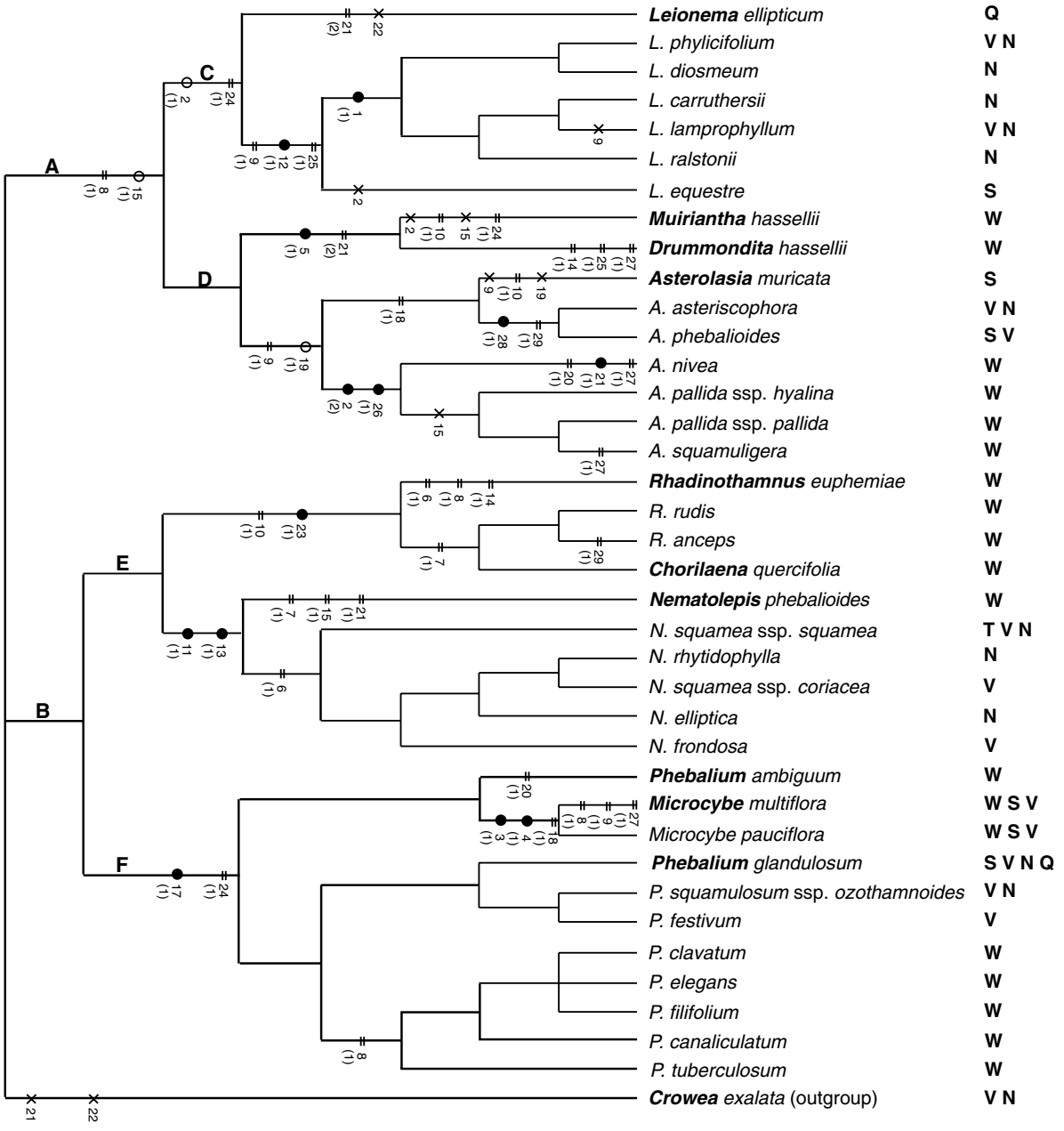

Fig. 4. Indel character transformations plotted on tree number one of the 90 most parsimonious trees. Solid dot indicates synapomorphy without reversal; cross (x) indicates a reversal; hollow dot indicates a synapomorphy with later reversal; parallel lines indicate a parallelism; numbers indicate indel numbers and numbers in parentheses indicate character states

The sister group to *Leionema* is clade D, node 4, containing *Muriantha, Drummondita* and *Asterolasia,* a grouping which lacks bootstrap support. Within clade D, *Muriantha* and *Drummondita* form a monophyletic group at node 12 (bootstrap support 60%), which is the sister group to *Asterolasia*, at node 13. The position of *Drummondita* is interesting since it was originally included in the analysis as one of the outgroup taxa. The relationships of *Drummondita* have been unclear. It was treated as a synonym of

Table 4. Insertion/deletion characters

Indel number	base position	size (bp)	indel type
1	74	1	insertion
2	76	1	insertion
3	80	1	insertion
4	111	1	deletion
5	119	1	deletion
6	128	1	deletion
7	147	1	insertion
8	421	1	insertion
9	470-471	1 & 2	insertion
10	472	1	insertion
11	474	1	insertion
12	475	1	insertion
13	479	1	deletion
14	483	1	deletion
15	488	1	insertion
16	520-521	2	insertion
17	589	1	deletion
18	590	1	deletion
19	630	1	insertion
20	634	1	insertion
21	635	1	deletion
22	639	1	deletion
23	640	1	deletion
24	642	1	deletion
25	644	1	deletion
26	648-649	2	insertion
27	654	1	deletion
28	662-663	1 & 2	insertion
29	672-673	2	deletion

Philotheca Rudge until its reinstatement by Wilson in 1971.

All exemplars of *Asterolasia* sampled form a monophyletic group (node 13, bootstrap support 84%), which is supported by a synapomorphic indel that later reverses and a parallelism (indel characters 19 and 9, Fig. 4). There is an east-west geographic split within *Asterolasia* with all four taxa sampled from Western Australia (*A. pallida* subsp. *pallida*, *A. pallida* subsp. *hyalina*, *A. nivea* and *A. squamuligera*) forming a clade (node 15, bootstrap support 100%) that is sister to a South Australian-Victorian-New South Wales clade (node 14, bootstrap support 73%) including *A. muricata*, *A. asteriscophora* and *A. phebalioides*. Node 15 is supported by two synapomorphic indels (indel characters 2, and 26, Fig. 4). The Western Australian clade is comprised of taxa previously included in the genus *Urocarpus* Harvey, which have a reduced carpel number (see Wilson 1971, 1980, 1987). Interestingly, *A. muricata*, which was also previously included in *Urocarpus*, and which also has a reduced carpel number, is the sister taxon to two eastern Australian *Asterolasia* species rather than to the Western Australian taxa.

Clade B. Clade B is comprised of the genera *Nematolepis, Rhadinothamnus, Chorilaena, Phebalium* and *Microcybe*. Within clade B (which lacks bootstrap support), *Nematolepis, Rhadinothamnus* and *Chorilaena* form a monophyletic group (clade E, node 5, bootstrap support 81%), which is sister to a monophyletic group containing *Phebalium* and *Microcybe* (clade F, node 6, bootstrap support 96%). Within clade E, *Nematolepis* forms a monophyletic group at node 18 with high bootstrap support (86%) and two indel synapomorphies (characters 11 and 13, Fig. 4). Resolution within a group of four eastern Australian taxa of *Nematolepis* at node 22 is low, indicating that they are not highly divergent and are closely related. *Nematolepis squamea* subsp. *squamea*, which is the sister group to the clade at node 22 (bootstrap support 71%), is the most divergent of the eastern *Nematolepis*. The clade at node 22 includes *N. squamea* subsp. *coriacea*, suggesting that *N. squamea* as currently circumscribed is not monophyletic, and that *N. squamea* subsp. *coriacea* should be recognised at specific rank. There is high bootstrap support (100%) at node 20 for an east – west geographic division in *Nematolepis* (a feature which is recurrent for other genera). *Nematolepis phebaliodes* (node 18) was previously classified as a monotypic genus and is currently the sole Western Australian representative of *Nematolepis* (Wilson 1998).

Nematolepis is the sister taxon (node 5, bootstrap support 81%) to a clade at node 17 (bootstrap support of 100%) that consists of *Rhadinothamnus euphemiae*, *R. rudis*, *R. anceps*

and *Chorilaena quercifolia*. Node 17 is supported by two indel characters: one synapomorphy and one parallelism (characters 23 and 10, Fig. 4). *Rhadinothamnus* is thus paraphyletic with respect to *Chorilaena*. *Rhadinothamnus anceps* and *R. rudis* form a monophyletic group (node 21), which is sister to *Chorilaena*. The results support Wilson (1998) who considered *Chorilaena, Rhadinothamnus* and *Phebalium* section *Gonioclados* to be closely related on the basis of shared characters such as the hilar strand, hemispherical calyx, valvate petals and non-glandular apiculum of the anthers.

Phebalium s. str. and *Microcybe* form a monophyletic group (Clade F, node 6, bootstrap support 96%) supporting Wilson's (1970, 1998) hypothesis of the close relationship of these taxa. This clade is also supported by two indel characters (character 17, synapomorphy and 25, parallelism, Fig. 4). The tree topology indicates that *Phebalium* is paraphyletic. *Microcybe* is clearly a monophyletic group at node 27 with a strong bootstrap support of 98% and two synapomorphic indels and one parallelism (characters 3, 4 and 18, Fig. 4). *Microcybe* and *Phebalium ambiguum* are sister taxa (node 23, bootstrap support 63%) and together are the sister group to a clade (node 24, bootstrap support 89%) comprised of all remaining *Phebalium* exemplars. *Phebalium ambiguum*, which is endemic to the Western Australian wheat-belt region, was originally described as a species of *Microcybe* by Herbert (1922), presumably on the basis of its habit and sessile flowers, but was later transferred to *Phebalium* by Gardner (1942). Gardner did not give any reason for his placement of the species in *Phebalium* and, as indicated by his choice of specific epithet, he had doubts about its placement in the genus. *Phebalium ambiguum* is morphologically distinct from all other species of *Phebalium* in possessing a reduced carpel number (three, rarely four and then the fourth much reduced in size), imbricate petals, which are almost valvate, and solitary sessile flowers. It can be distinguished from *Microcybe* by the carpel number (two in *Microcybe*), lepidote petals, and the solitary flowers. With the exclusion of *P. ambiguum*, *Phebalium* has an east-west geographic split with a western clade (node 26) comprising *P. clavatum*, *P. elegans*, *P. filifolium*, *P. canaliculatum* and *P. tuberculosum*, and a sister group (node 25) comprising the eastern species *P. glandulosum*, *P. squamulosum* subsp. *ozothamnoides* and *P. festivum*. *Phebalium squamulosum* subsp. *ozothamnoides* and *P. festivum* are sister taxa with a bootstrap support of 53% (node 28).

Discussion

Generic relationships. With respect to generic relationships within the *Phebalium* group, ITS sequences have proved informative, although difficult to obtain. The results support some of the findings of Wilson (1970, 1971, 1998) and disagree with others. He suggested a relationship between *Rhadinothamnus, Nematolepis, Chorilaena* and *Muiriantha* and this is supported by the inclusion of three of these taxa in our clade E. Wilson (1970) suggested also a relationship between the eastern Australian *Nematolepis* (then *Phebalium* section *Eriostemonoides*) and *Asterolasia*, which is not supported by the ITS data. *Asterolasia* is in clade A with *Leionema, Muiriantha* and *Drummondita*, although this grouping lacks bootstrap support, while *Nematolepis* is in clade B. The ITS cladogram shows *Rhadinothamnus* (previously *Phebalium* section *Gonioclados*, *Nematolepis* (section *Eriostemoides*) and *Phebalium* (section *Phebalium*) all as members of clade B. *Phebalium* and *Microcybe* (clade F, node 6) form a monophyletic group, which supports Wilson's conclusions that these two genera are closely related (Wilson 1970, 1998).

Taxonomic implications. The results largely support the recent division of *Phebalium* into four separate genera (Wilson 1998), although some additional taxonomic revision appears necessary. *Rhadinothamnus* is paraphyletic with respect to *Chorilaena*. *Rhadinothamnus anceps* and *R. rudis* are sister taxa (although lacking bootstrap support) and were formerly recogni-

sed as the taxon *Phebalium* section *Gonioclados* (Wilson 1970). Together, they form a monophyletic group with *Chorilaena* that is sister to *R. euphemiae*, the type for *Rhadinothamnus*. This suggests that either *Rhadinothamnus* be transferred to *Chorilaena*, or that *R. anceps* and *R. rudis* (the former members of *Phebalium* section *Gonioclados*) be given generic status. Transferring *Rhadinothamnus* to *Chorilaena* would result in an extremely morphologically diverse genus, and since *Phebalium* section *Gonioclados* has previously been recognised as a discrete taxon, the prefered option is to raise it to generic rank.

Based on morphological differences and the relationships inferred from the molecular data, *Phebalium ambiguum* also warrants recognition at generic level. One alternative would be to accept a broader concept of *Phebalium* by transferring *Microcybe* to *Phebalium*, a course of action that has previously been considered and rejected on morphological grounds by Wilson (1998). Another alternative would be to accept a broader concept of *Microcybe* by transferring *Phebalium ambiguum* to *Microcybe*.

Muiriantha and one of the original outgroups, *Drummondita*, are identified as sister taxa, however, the inclusion of additional species of *Drummondita* is required before any further discussion of this relationship can be made. All other genera included in the analysis (*Nematolepis, Leionema, Asterolasia* and *Microcybe*) are monophyletic and should be retained. In *Asterolasia* there is some support for the recognition of a taxon, possibly at sectional rank, that contains many of the Western Australian species previously placed in the genus *Urocarpus*. Further sampling is required to determine if this classification is warranted. The main morphological feature once used to segregate the genera, carpel number, does not support the division (see also Wilson 1971, 1980, 1987) as *A. muricata*, with a reduced carpel number, is sister to eastern species that have five carpels.

A further taxonomic change to be considered is the recognition of *Leionema ellipticum* as either a monotypic genus or a separate section within *Leionema*. *Leionema* is monophyletic but with *L. ellipticum* from northern Queensland sister to all other taxa sampled (eastern states of Australia and South Australia). The South Australian *L. equestre* may also warrant sectional rank within *Leionema*.

Formal taxonomic changes will be dealt with after detailed analysis of morphological characters in combination with the molecular results of this study and inclusion of exemplar taxa that were unable to be sequenced.

Biogeography. Burbidge (1960), Croizat (1962) and others (e.g. Crisp et al. 1995, Ladiges 1998) have discussed the historical phytogeography of Australia in relation to dispersal and/or vicariance events. The *Phebalium* group, with its endemic taxa, is suitable for biogeographic analysis, and should contribute to a greater understanding of historical area relationships in Australia and between Australia and New Zealand.

Based on the taxa able to be sequenced in this ITS study, the phylogenetic tree (Fig. 2) shows geographic patterns that are congruent with other findings. Three of the genera in the *Phebalium* group have sister clades in eastern and south-western Australia (*Phebalium, Nematolepis* and *Asterolasia*). This repeated east-west pattern (geographic paralogy) is suggestive of lineages that existed prior to vicariance events that led to the isolation and differentiation of these major regions. The relative ages of vicariance events

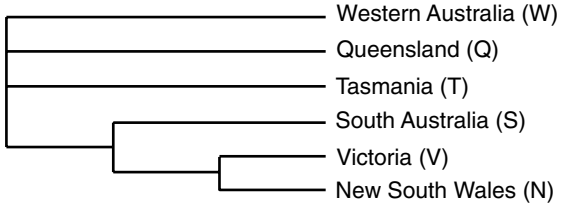

Fig. 5. Summary of geographic area relationships derived from the taxon cladogram (Fig. 2) based on the method of subtree analysis (Nelson and Ladiges 1996). Note that no taxa endemic to Tasmania and New Zealand were able to be sequenced

is summarised in an area cladogram (Fig. 5) based on subtree analysis and Assumption 2 (Nelson and Ladiges 1996) of the taxon consensus tree (Fig. 2). The geographic regions are: W south-west Western Australia; T Tasmania; S South Australia; V Victoria, N New South Wales, Q Queensland. Clade C of the taxon tree gives the area subtree Q (S (V, N)); clade D subtree W (S (V, N)); clade E subtree W (T (V, N)); and clade F subtree W (S (V, N). Combining these subtrees gives the summary area cladogram (Fig. 5), which indicates that the isolation of south-west Western Australia, northern Queensland and Tasmania from the other regions are the earliest events, followed by South Australia and then Victoria and New South Wales. Burbidge (1960) believed Rutaceae to be a relatively old group within the endemic Australian flora. Isolation of western and eastern regions in southern Australia may thus relate to marine transgressions (Eocene and Miocene) and/or climate change with increased aridity (from the Oligocene). Repeated waves of dispersal from west to east or east to west is a less parsimonious explanation.

Leionema has an eastern distribution in Australia (Tasmania, Victoria, South Australia, New South Wales, and Queensland) and includes one New Zealand species, *L. nudum*. The pattern of *L. ellipticum* from tropical Queensland in a basal position, and *L. equestre* from Kangaroo Island (South Australia) as sister group to the other eastern regions, is similar to other area relationships based on Australian taxa (see Ladiges 1998). It is impossible to speculate on the eastern Australia-New Zealand connection without the sequence data for the New Zealand species.

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