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ORIGINAL PAPER

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Chloroplast DNA inheritance and variation in *Leucadendron* species (Proteaceae) as revealed by PCR-RFLP

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Abstract The inheritance of chloroplast DNA (cpDNA) in *Leucadendron* species was studied by polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) analysis. A total of 100 progeny from five interspecific crosses involving seven parental species were tested, and all progeny exhibited the cpDNA restriction fragment pattern of the female parent, indicating that cpDNA in *Leucadendron* is maternally inherited. PCR-RFLP was also employed to study cpDNA variation among 21 *Leucadendron* species. Parsimony analysis using a heuristic search resulted in a phylogenetic tree that showed limited agreement to the taxonomic classification of *Leucadendron* species, based on morphological characteristics. The incongruence between cpDNA phylogenetic and taxonomic groupings in *Leucadendron* may be due to reticulate evolution involving a combination of hybridization and introgression, convergent evolution and/or lineage sorting at the interspecific, intersubsectional and intersectional levels.

Introduction

Members of the genus *Leucadendron* (Proteaceae) are endemic to fynbos shrub lands of the Cape Floristic region of South Africa (Vogts 1982). In Western Australia, *Leucadendron* species are grown extensively because of the importance of the stems to the cutflower industry. The genus contains 80 species, which have been classified into two sections by Williams (1972), based on morphological fruit characteristics. Section *Leucadendron* has 47 species and three subspecies with rounded, nut-like fruits, while section *Alatosperma* includes the 33 species with flattened fruits. Williams (1972) further divided section *Leucadendron* into ten subsections and section *Alatosperma* into four subsections, according to additional characteristics of the fruit or seed.

The availability of molecular markers for *Leucadendron* would be useful in assisting parent selection in breeding programs and could be used as a tool to identify the parentage of natural collections as well as to study the gene flow and evolution of this genus. Chloroplast DNA (cpDNA) markers have been applied extensively to study relationships among plants. Unlike the nuclear genome, cpDNA shows uniparental inheritance in most plant species (Birky 1995; Reboud and Zeyl 1994), does not undergo recombination and has a slow rate of sequence and structural evolutionary change (Palmer et al. 1988). Specific amplification of cpDNA fragments, using polymerase chain reaction (PCR), followed by restriction fragment length polymorphism (RFLP) analysis of the amplification products, has been applied extensively to examine cpDNA variation (Cipriani et al. 1998; Parducci and Szmidt 1999) and to determine the mode of organelle inheritance (Chat et al. 1999). It has also been used to identify seed parents in *Iris* (Arnold et al. 1991) and to reveal phylogenetic relationships in numerous genera (Perez de la Rosa et al. 1995; Isshiki et al. 1998).

Chloroplast DNA is maternally inherited in most angiosperms, although cpDNA has a strict paternal inheritance in kiwifruit (Chat et al. 1999) and is paternally transmitted with high frequency in alfalfa (Masoud et al. 1990). In contrast, cpDNA is paternally inherited in most gymnosperms (Mogensen 1996). We have used PCR-RFLP to show that cpDNA is maternally inherited in *Leucadendron* species, and we have used cpDNA variation to examine the relationships among *Leucadendron* species.

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Materials and methods

Plant materials

Leaf tissue from one representative of 21 *Leucadendron* species, the progeny of five interspecific crosses of *Leucadendron*, and *Leucospermum cordifolium* were sampled from the living collections of the *Leucadendron* breeding program at the University of Western Australia (Tables 1, 2).

DNA extraction and analysis

Total DNA was extracted from approximately 0.1 g of leaves, using the DNeasy Plant Mini Kit (Qiagen, Clifton Hill, VIC, Australia) according to the manufacturer's instructions. DNA was examined electrophoretically on agarose gels stained with ethidium bromide (Sambrook et al. 1989). The concentration of DNA was estimated by comparison to a λDNA mass marker (MBI, Fermentas, Hanover, MD., USA).

PCR-RFLP

The oligonucleotide primers (Life Technologies, Mount Waverley, VIC, Australia) used in this study (Table 3) are universal for the PCR amplification of land-plant cpDNA (Demesure et al. 1995; Dumolin et al. 1997). The PCR conditions, optimized to avoid formation of non-specific products, were 20 ng DNA template, 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 0.1% Triton X-100, 1.5 mM $MgCl_2$, 0.3 μM each primer, 200 μM each dNTP and 1 U *Taq* DNA polymerase (Promega, Annandale, NSW, Australia) in 25-μl reactions. Amplification of PCR products larger than 2 kb was carried out using Bio*Taq* DNA polymerase (Bioline, Alexandria, NSW, Australia) and 16 mM $(NH_4)_2SO_4$, 67 mM Tris-HCl (pH 8.8), and 0.01% Tween 20. The amplification conditions used were initial denaturation at 94°C for 4 min, followed by 30 cycles of 45 s at 94°C, 45 s at 53.5–62°C (depending on the primers used, Table 3) and 2 or 3 min at 72°C (Table 3), with a final 10 min extension cycle at 72°C. In every experiment a negative control containing all components except DNA template (replaced by water) was included to test reagents for DNA contamination. The amplicons were separated in 1.5% agarose gels stained with ethidium bromide. Aliquots of the amplicons were digested at 37°C for 3 h with 2 U of *Alu*I, *Cfo*I, *Hae*III, *Nde*II or *Eco*RV (Promega), or *Mva*I, *Msp*I or *Hin*dfI (Roche Diagnostic, Castle Hill, NSW, Australia) in 10-μl reactions containing 1× buffer supplied by the enzyme manufacturer.

Data analysis

DNA gels were photographed (Kodak Digital Science1D, Eastman Kodak, Rochester, N.Y., USA), and the sizes of the fragments were determined (Kodak Digital Science 1D Image Analysis Software, Eastman Kodak). Restriction site polymorphisms were identified using site occurrence analysis (Bremer 1991). The presence or absence of restriction sites was scored as 1 or 0, respectively. Length polymorphisms were identified using the outgroup comparison method (Watrous and Wheeler 1981; Pillay and Hilu 1995). In both analyses, *Leucospermum cordifolium* was chosen as an outgroup because of the close relationship of *Leucospermum* to *Leucadendron* (Rourke 1998). A data matrix of the characters was subjected to Wagner and Dollo analyses [phylogenetic analysis using parsimony (PAUP), version 4.0, Swofford 1998], using a heuristic search with a random addition sequence of 100 replicates and TBR branch swapping with the steepest descent option selected.

Results

Chloroplast DNA inheritance

Amplification of cpDNA fragments using SM, HK, ST and KK primer pairs (Table 3) failed to detect length polymorphisms among the parents of five interspecific crosses of *Leucadendron*. Therefore, PCR–RFLP experiments were conducted. Several primer–enzyme combinations (SM, digested with *Eco*RV, *Cfo*I, *Nde*II, *Alu*I or *Hae*III; ST, digested with *Eco*RV, *Cfo*I, *Nde*II, *Alu*I or *Hae*III; HK, digested with *Eco*RV or *Hae*III; KK, digested with *Eco*RV, *Cfo*I or *Nde*II) failed to reveal any polymorphisms. However, digestion of the KK amplification product with *Hae*III or *Alu*I (Table 1) revealed polymorphisms among parental pairs. The *L. salignum* and

Table 1 Inheritance of chloroplast DNA (cpDNA) in *Leucadendron* interspecific hybrids. The cpDNA fragment analysed was amplified using the KK primer pair (Table 3)

Cross [parents (♀ × ♂)]	Restriction enzyme	Maternal fragment pattern (bp)	Paternal fragment pattern (bp)	No. of offspring with fragment		
				Maternal	Paternal	Biparental
L. gandogeri 06 × *L. procerum* 04	*Hae*III	1,245/720/645	1,965/645	21	0	0
L. eucalyptifolium 03 × *L. procerum* 04	*Hae*III	1,245/720/645	1,965/645	18	0	0
L. uliginosum 05 × *L. procerum* 04	*Hae*III	1,245/720/645	1,965/645	19	0	0
L. laureolum 04 × *L. procerum* 04	*Hae*III	1,245/720/645	1,965/645	22	0	0
L. salignum 11 × *L. discolor* 02	*Alu*I	950/675/390/310/285	1,625/390/310/285	20	0	0

Table 2 The *Leucadendron* species studied and their classification

No.	Species	Accession	Section[a]	Subsection[a]	Haplotype[b]
1	*L. argenteum* R.Br.	LAC 30	*Leucadendron*	*Leucadendron*	I
2	*L. linifolium* R.Br.	LLE	*Leucadendron*	*Villosa*	II
3	*L. galpinii* Phillips and Hutchinson	LGA 07	*Leucadendron*	*Villosa*	IX
4	*L. tinctum* I.Williams	LTC 01	*Leucadendron*	*Nucifera*	X
5	*L. salicifolium* (Salisb.) I.Williams	LSA 01	*Alatosperma*	*Trigona*	III
6	*L. conicum* (Lam.) I.Williams	LCF 01	*Alatosperma*	*Trigona*	VII
7	*L. uliginosum* R.Br.	LUA 05	*Alatosperma*	*Trigona*	VII
8	*L. floridum* R.Br.	LFB 05	*Alatosperma*	*Trigona*	XI
9	*L. macowanii* Phillips	LMA 001	*Alatosperma*	*Trigona*	XII
10	*L. flexuosum* I.Williams	LFA 21	*Alatosperma*	*Alata*	IV
11	*L. coniferum* Meisn.	LCG 02	*Alatosperma*	*Alata*	V
12	*L. meridianum* Salter ex I.Williams	LMB	*Alatosperma*	*Alata*	V
13	*L. stelligerum* I.Williams	LSK 01	*Alatosperma*	*Alata*	V
14	*L. eucalyptifolium* Buex ex Meisn.	LEC 03	*Alatosperma*	*Alata*	VI
15	*L. gandogeri* Schinz ex Gand.	LGB 06	*Alatosperma*	*Alata*	VI
16	*L. spissifolium* (Salisb. ex Knight) I.Williams	LSI 01	*Alatosperma*	*Alata*	VI
17	*L. salignum* Berg.	LSB 11	*Alatosperma*	*Alata*	VIII
18	*L. strobilinum* Druce	LSL 02	*Alatosperma*	*Alata*	XIII
19	*L. procerum* (Salisb. ex Knight) I.Williams	LPB 04	*Alatosperma*	*Alata*	XIV
20	*L. laureolum* (Lam.) Fourc.	LLB 04	*Alatosperma*	*Alata*	XV
21	*L. muirii* Phillips	LMF	*Alatosperma*	*Compressa*	XVI
22	*L. discolor* Buek ex Meisn.	LDC 02	*Alatosperma*	*Alata*	
23	*Leucospermum cordifolium* (Knight) Fourc.				

[a]According to Williams (1972)
[b]This study

L. discolor parental cpDNA fragments were differentiated by digestion with *Alu*I (Table 1). The *L. procerum* paternal fragment was differentiated from the *L. laureolum*, *L. uliginosum*, *L. eucalyptifolium* and *L. gandogeri* maternal fragments by digestion with *Hae*III (Table 1).

To investigate cpDNA inheritance in *Leucadendron*, 19 progeny from a *L. uliginosum* × *L. procerum* cross were examined by PCR-RFLP. All the progeny contained the cpDNA pattern of the maternal parent (Fig. 1). Identical results were found for the other four interspecific crosses tested (Table 1). In all, 100 progeny were analysed for cpDNA parentage (Table 1), with all offspring showing the maternal cpDNA pattern.

Chloroplast DNA variation and phylogeny of *Leucadendron*

Seven primer pairs (Table 3) were used to study cpDNA variation in 21 species of *Leucadendron*, with *Leucospermum cordifolium* as an outgroup. All primer pairs successfully amplified a single fragment from the total DNA of the species tested (Table 3) but failed to reveal length polymorphisms. Variations in fragment pattern among species were found when the 3,050-bp PCR product amplified with the FV primer pair was digested with *Hae*III (Fig. 2). In total, 24 of the 33 enzyme–primer pair combinations tested showed polymorphic patterns. Seven of these combinations were excluded, because they revealed length polymorphisms that were identical to those

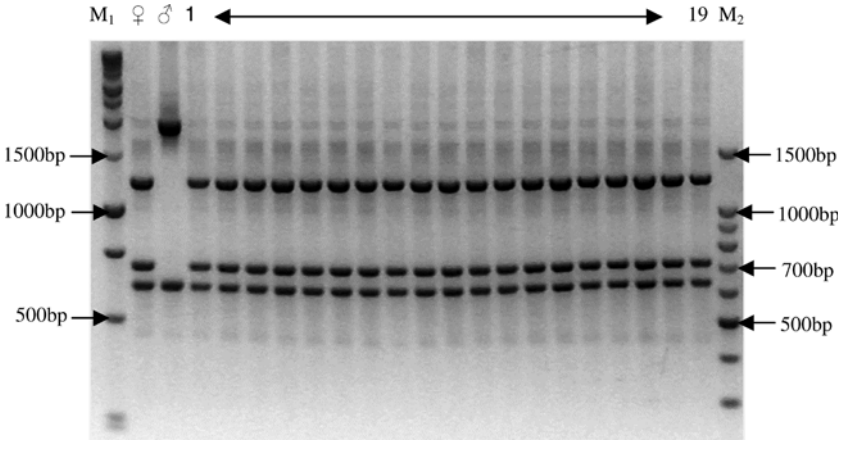

Fig. 1 Digestion pattern of chloroplast DNA (cpDNA) fragments from progeny of *Leucadendron uliginosum* (♀) × *L. procerum* (♂). A cpDNA fragment was amplified using the KK primer pair from 19 progeny (*lanes 1–19*). The *Hae*III digestion products were separated on a 3% agarose gel stained with ethidium bromide. *Lane* M_1 contains a 1-kb ladder, and *lane* M_2 contains a 100-bp ladder. Sizes of selected marker fragments are shown on the *left* and *right* of the figure

Table 3 Primers used in the present study and the size of the observed products

Primer pair	Abbreviation	Sequence	Annealing temperature (°C)[a]	Extension time (min)[a]	Product size (bp)	References
trnF	FV	5′-CTC GTG TCA CCA GTT CAA AT-3′	58	3	3,050	Dumolin et al. (1997)
trn Vr		5′-CCG AGA AGG TCT ACG GTT CG-3′				
trnV	VL	5′-CGA ACC GTA GAC CTT CTC GG-3′	59	3	3,890	Dumolin et al. (1997)
rbcLr		5′-GCT TTA GTC TCT GTT TGT GG-3′				
trnH[tRNA-His(GUG)]	HK	5′-ACG GGA ATT GAA CCC GCG CA-3′	62	2	1,755	Demesure et al. (1995)
trnK[tRNA-Lys(UUU)exon1]		5′-CCG ACT AGT TCC GGG TTC GA-3′				
trnK[tRNA-Lys(UUU)exon1]	KK	5′-GGG TTG CCC GGG ACT CGA AC-3′	53.5	3	2,610	Demesure et al. (1995)
trnK[tRNA-Lys(UUU)exon2]		5′-CAA CGG TAG AGT ACT AGG CTT TTA-3′				
trnS[tRNA-Ser(UGA)]	SM	5′-GAG AGA GAG GGA TTC GAA CC-3′	62	2	1,175	Demesure et al. (1995)
trnfM[tRNA-fMet(CAU)]		5′-CAT AAC CTT GAG GTC ACG GG-3′				
psaA[PS I (P700 apoprotein A1)]	AS	5′-ACT TCT GGT TCC GGC GAA CGA A-3′	59	3	3,050	Demesure et al. (1995)
trnS[tRNA-Ser(GGA)]		5′-AAC CAC TCG GCC ATC TCT CCT A-3′				
trnS[tRNA-Ser(GGA)]	ST	5′-CGA GGG TTC GAA TCC CTC TC-3′	57.5	2	1,270	Demesure et al. (1995)
trnT[tRNA-Thr(UGU)]		5′-AGA GCA TCG CAT TTG TAA TG-3′				

[a]Empirically determined to prevent amplification of non-specific DNA fragments

found when the same amplicon was digested with other enzymes. It is appropriate to score such length mutations as only single mutational events (Byrne et al. 2001). From the remaining 17 combinations, a total of 47 characters were identified (Table 4). The 47 characters, which included 15 site mutations and 32 length mutations, produced 16 haplotypes (Table 2). Several *Leucadendron* species were found to have the same haplotype: *L. coniferum*, *L. meridianum* and *L. stelligerum* have haplotype V; *L. eucalyptifolium*, *L. gandogeri* and *L. spissifolium* have haplotype VI; and *L. conicum* and *L. uliginosum* have haplotype VII.

The 47 polymorphic characters were used in Wagner analysis to reconstruct the *Leucadendron* phylogeny. The five equally most parsimonious trees, each with a length of 55 steps, a consistency index of 0.855, a retention index of 0.900 and a homoplasy index of 0.145, were used to generate a strict consensus tree (Fig. 3). Ten species were well separated on the tree and were assigned to clade A. *Leucadendron galpini* and *L. tinctum* were assigned to clade B, and were well separated from *L. floridum*, *L. macowanii* and *L. strobilinum*, which formed clade C. The *L. muirii* lineage was distinct from the main *Leucadendron* lineage. The branching order for the remaining five species could not be resolved.

The PCR–RFLP character set was also subjected to Dollo parsimony analysis. The most parsimonious tree resulting from Dollo analysis had more steps (61), a lower consistency index (0.771) and a higher homoplasy index (0.229) than the trees from the Wagner analysis. The Dollo tree had similar topology to the strict consensus tree from the Wagner analysis and recognized both clades A and B. The main difference between the trees was that clade C identified by Wagner analysis (Fig. 3) was embedded within clade A by the Dollo analysis and split from the other species of clade A at the level of *L. eucalyptifolium*, *L. gandogeri* and *L. spissifolium*. The position of *L. laureolum* was also shifted to within clade A, grouping closely with *L. salicifolium*.

Discussion

Chloroplast DNA inheritance

The cpDNA in the majority of angiosperms is maternally inherited, although there are several exceptions (Lee et al. 1988; Chat et al. 1999). Using a PCR–RFLP-based approach, we demonstrate for the first time, that cpDNA in a member of the Proteaceae, *Leucadendron*, is likely to be maternally inherited. This conclusion is based on the finding that all 100 progeny examined from five interspecific crosses exhibited the maternal pattern of cpDNA. To assess the validity of our finding, we used the binomial model of Milligan (1992), which evaluates the probability of transmission (P) from the alternative (paternal) parent by the following relationship: $P=1-(1-\beta)^{1/n}$ where n is the size of sample and $(1-\beta)$ is the probability of falsely accepting the strictly maternal hypothesis. With $n=100$

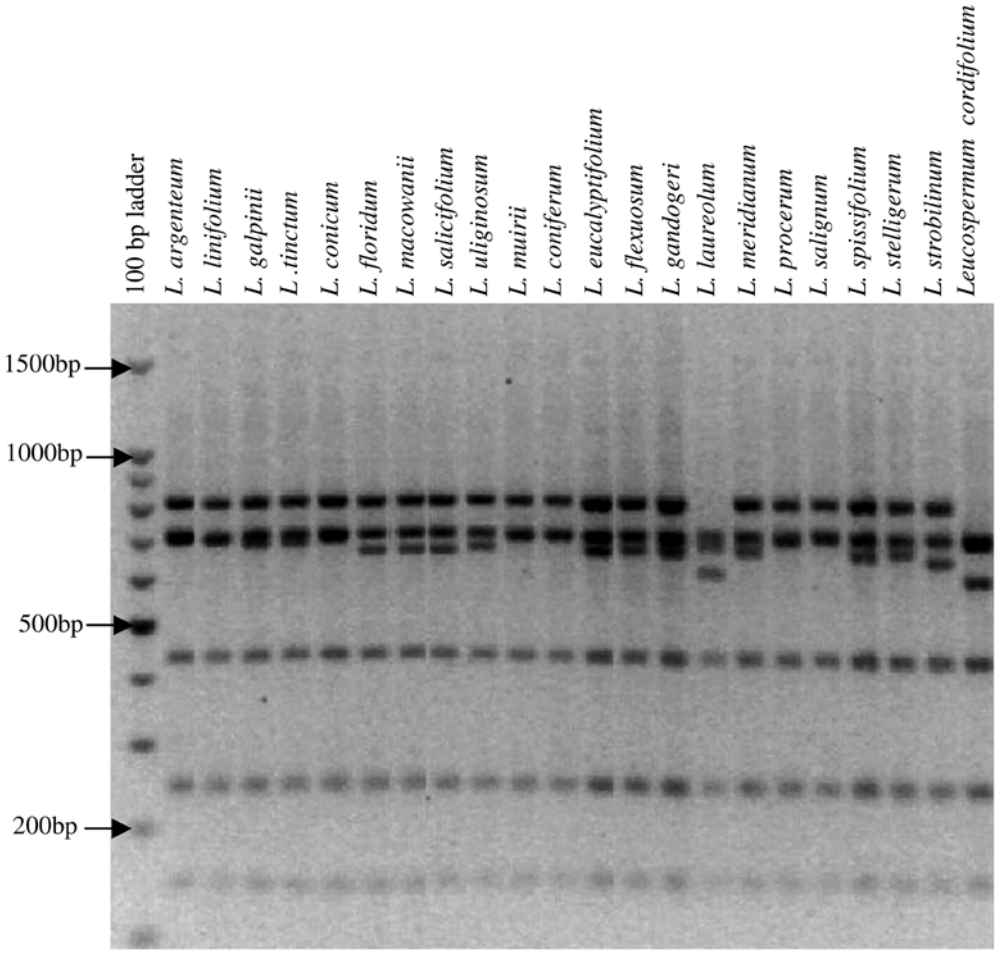

Fig. 2 Variation in cpDNA from *Leucadendron* species revealed using the FV–*Hae*III primer–enzyme combination. The digested cpDNA fragments were separated on a 3% agarose gel stained with ethidium bromide. The species tested are shown across the *top of the figure*. The sizes of selected marker fragments are shown on the *left of the figure*

Table 4 The polymerase chain reaction–restriction fragment length polymorphism characters identified in cpDNA of *Leucadendron* species

Character	Primer pair-enzyme	Mutation (bp)[a,b]	Character	Primer-pair enzyme	Mutation (bp)[a,b]
1	HK-*Hin*dfI	715→740	24	FV-*Nde*II	500→490
2		715→724	25		500→465
3	HK-*Cfo*I	725=465+260	26		500→480
4	FV-*Mva*I	1,060→850+*x*	27		500→445
5	FV-*Alu*I	530→355+*x*	28		210→*x*+*y*
6		840→830	29		230→225
7		840→810	30	VL-*Cfo*I	1,220→1,250
8		840→815	31	AS-*Alu*I	835→870
9		840→835	32		835→910
10	KK-*Hae*III	1,245+720=1,965	33		835→860
11		365+280=645	34		835→800
12	KK-*Alu*I	1,625+310=1,935	35	AS-*Hae*III	850→830
13		1,625=950+675	36		850→935
14	ST-*Mva*I	1,270=730+540	37		850→920
15	SF-*Msp*I	210→200	38		850→950
16	VL-*Mva*I	1,080+670=1,750	39	AS-*Cfo*I	2,250=600+1,650
17	SF-*Hae*III	810→555+255	40		535→600
18	FV-*Hae*III	600+*x*=825	41		535→575
19		625+*x*=825	42		535→635
20		730→660	43	AS-*Mva*I	840=640+200
21		730→685	44		840→850
22		730→700	45		840→970
23		730→725	46		840→890
			47		840→835

[a]Compared to *Leucospermum cordifolium*
[b]Fragments not detected are designated *x* and *y*

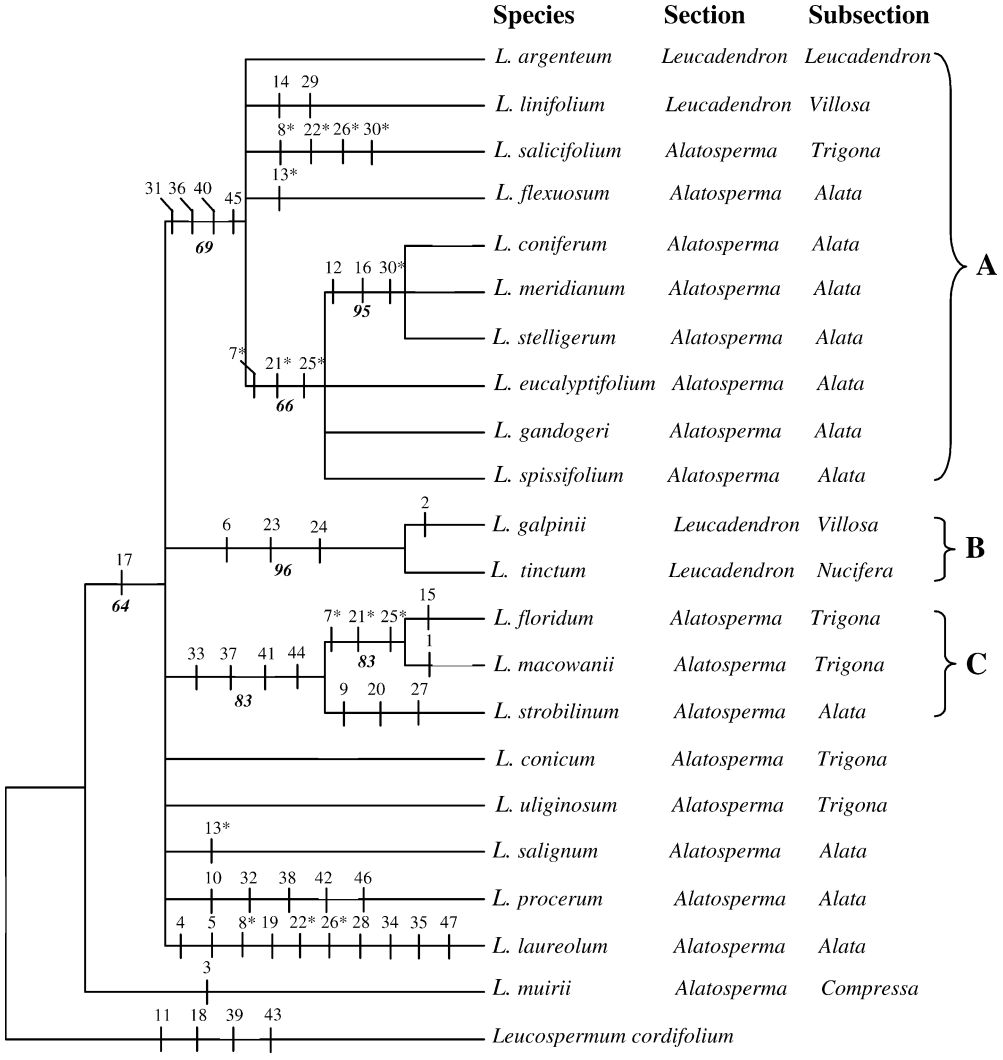

Fig. 3 Relationships of *Leucadendron* species, based on the analysis of cpDNA variation. Following the name of each species are its section and subsection. (Williams 1972). *Numbers above the lines* indicate the characteristics that have changed (Table 4). *Asterisks* indicate homoplasy. Bootstrap values >50% are shown *below the lines*.

progeny analysed and β=0.95, the probability of paternal transmission in this study is 0.03. Therefore, it is highly likely that cpDNA is maternally inherited in *Leucadendron*.

Chloroplast DNA variation and phylogenetic implications of the cpDNA cladogram

In the PCR–RFLP analysis used to study cpDNA variation among 21 *Leucadendron* species, no length polymorphisms were detected in the PCR products amplified using any of the primer pairs tested. However, numerous restriction site polymorphisms were found in the amplified products. Using 33 primer pair–enzyme combinations, we detected 32 length and 15 restriction site polymorphisms among the species tested. The preponderance of length polymorphisms is consistent with studies that demonstrated that most of the observed cpDNA variation in the genus *Abies* and in conifers was due to length polymorphisms (Hipkins et al. 1994; Parducci and Szmidt 1999).

The cpDNA data were used to produce the first reconstruction of the *Leucadendron* phylogeny (Fig. 3). Comparison of the cpDNA phylogeny with the taxonomic classification of *Leucadendron* based on morphological data (Williams 1972; Midgley 1987) showed several apparent discrepancies. Species grouped into section *Leucadendron* by Williams (1972) were well distinguished from each other on the basis of cpDNA characters into clades A and B with moderate bootstrap confidence. The separation of section *Leucadendron* extended to the subsection level, with *L. linifolium* and *L. galpinii*, both previously assigned to subsection *Villosa*, being separated into clades A and B, respectively. The five *Alatosperma* species within subsection *Trigona* did not show significant phylogenetic affinity to one another except *L. floridum* and *L. macowanii*. It is possible that *L. conicum* and *L. uliginosum* may have an affinity, but this was not clear from the cpDNA analysis. Our analysis showed that *L. muirii* is distinct from the other species examined.

The incongruence between cpDNA-based phylogenies and morphology-based taxonomies has been reported widely (Soltis et al. 1991; Steane et al. 1998; Cipriani et al. 1998; McKinnon et al. 1999). It is worth noting that the taxonomic classification of Williams (1972) is based on morphological characters that are assumed to be inherited

largely biparentally. In this case, hybridization between two individuals that differ morphologically and genetically would lead to offspring that are intermediate for the majority of morphological characters (Whittmore and Schaal 1991). In contrast, the cpDNA in the hybrids would be identical to that of the maternal parent, due to apparent strict maternal mode of cpDNA inheritance in *Leucadendron*. This situation may lead to hybrids that are morphologically distinct from the parent, but have the same cpDNA haplotype as the maternal parent.

The mechanisms that could cause morphologically distinct *Leucadendron* species to have similar cpDNA haplotype are diverse. An interspecific hybridization event may have led to cpDNA introgression from one parent species to the other. This scenario has been proposed by Soltis et al. (1991) to explain cpDNA distribution in *Heuchera* and may have been driven by a process such as variation in fertility between species, as has been proposed to explain the cpDNA distribution in cottonwood (Martinsen et al. 2001). This mechanism will result in species unrelated at the nuclear gene level having similar cpDNA haplotypes (van Raamsdonk et al. 1997). The idea that cpDNA introgression occurs in *Leucadendron* is supported by the observation of successful hybridization within and between subsections (Williams 1972; Midgley 1987). Moreover, extensive interspecific hybridization within this genus has been accomplished during breeding programs and has produced fertile hybrids (Yan et al. 2001a, b).

The cpDNA of *L. muirii* was found to be highly divergent from the other *Leucadendron* species tested, suggesting an inability to form interspecific hybrids. This conclusion is supported by hybridization studies of Yan et al. (2001b), where artificial hybridization between *L. muirii* as female parent and *L. laureolum*, *L. salignum*, *L. procerum*, *L. salicifolium*, *L. linifolium* or *L. conicum* resulted in the failure of pollen-tube penetration and growth to the ovary end of the style. In many cases, the pollen did not germinate on the stigma.

The conclusion that interspecific hybridization may underlie the discrepancy between the observed phylogenetic relationship and the taxonomic classification implies that the original parental species were geographically interspersed (sympatric speciation). Our data are consistent with this hypothesis. The cpDNA analysis showed that *L. floridum* (*Trigona*) and *L. macowanii* (*Trigona*) are more closely aligned to *L. strobilinum* (*Alata*), forming a small group (clade C in Fig. 3), than to *L. salicifolium* (*Trigona*) with high bootstrap support (83%). In contrast, based on morphology, Williams (1972) suggested that *L. macowanii* is closely related to *L. salicifolium* (Trigona). Referring to their original distribution in South Africa, *L. floridum, L. macowanii* and *L. strobilinum* are endemic and restricted in the Cape Peninsula area, providing the opportunity for hybridization and the subsequent interspecific crosses necessary to effectively transfer cpDNA from one species to another. *L. salicifolium* on the other hand, is not found in this region (Fig. 4; Williams 1972; Rebelo 1995) and so would not have access to the cpDNA found in species of the Cape Peninsula. Haplotype V (Table 2) is common to *L. coniferum, L. meridianum* and *L. stelligerum* (all in section *Alatosperma*, subsection *Alata*), species with overlapping areas of distribution in southern coastal areas (Fig. 4; Williams 1972; Rebelo 1995). The latter three species have some similarity in their flowering time (Rebelo 1995), providing opportunity for interspecific hybridization. Finally, *L. conicum* and *L. uliginosum* (both in subsection *Trigona*), which share haplotype VII, also have similar distributions (Fig. 4).

Our analysis is unable to exclude that other processes underlie the incongruences between cpDNA phylogeny and morphological classification. The phylogenetic grouping of cpDNA from *Leucadendron* species of different morphological subsections (eg. species in clades B and C in Fig. 3) could also have arisen through lineage sorting (Neigel and Avise 1986). In this case, the cpDNA haplotypes found, for example, in clade C would have been found within the common ancestor that gave rise to both the *Trigona* and *Alata* subsections. A third possibility is that convergent evolution of chloroplast genomes in morphologically distinct species may have occurred.

The maternal inheritance of cpDNA and the phylogenetic relationships revealed by the cpDNA variation will be useful in designing crosses for future breeding programs. The cpDNA analysis will also provide a starting point for more detail phylogenetic analysis of the genus *Leucadendron*.

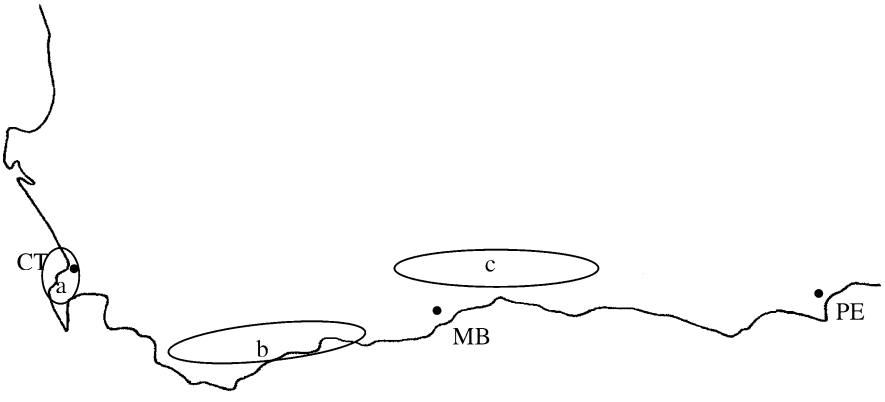

Fig. 4 Map of Fynbos Biome of South Africa showing the distribution of *L. floridum*, *L. macowanii* and *L. strobilinum* (**a**), *L. coniferum*, *L. meridianum* and *L. steligerum* (**b**), and *L. conicum* and *L. uliginosum* (**c**). *CT* Cape Town, *MB* Mossel Bay, *PE* Port Elizabeth

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