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Molecular analysis of interspecific and intergeneric relationships of *Banksia* using RAPDs and non-coding chloroplast DNA sequences

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Abstract Random amplified polymorphic DNA (RAPD) and chloroplast DNA (cpDNA) markers were used to study interspecific and intergeneric relationships of *Banksia* (Proteaceae) to aid breeding of the genus for cut flower production. The accepted morphological phylogeny of *Banksia*, with two subgenera, two sections and 13 series, is unclear regarding the relationships of the commercial cut flower species *B. coccinea*. Fifteen RAPD primers and a non-coding cpDNA sequence between the *trn*L (UAA) and *trn*F (GAA) gene were applied to species of *Banksia*, the related genus *Dryandra*, and to *Musgravea heterophylla* as the outgroup, with cluster analysis applied to the results. The two methods were in broad agreement with each other, and with the accepted taxonomy, with closely related species pairs and groups clustering together, but RAPDs were not informative between distantly related species or species pairs. *Banksia coccinea* clustered with *Dryandra* and formed a polytomy with 2 *Dryandra* species and the two sections of subgenus *Banksia*. Subgenus *Isostylis* formed a polytomy with *D. formosa*, basal to subgenus *Banksia*, but with *B. cuneata* and *B. illicifolia* (both in subgenus *Isostylis*) polyphyletic. *Dryandra* did not separate as a clade and fell within *Banksia*, raising questions about the currently accepted view of the two as sister genera with parallel morphological development. The results indicate that interspecific and intergeneric hybridisation with genus *Dryandra* and subgenus *Banksia* may be possible routes for improvement of the commercial species *B. coccinea*.

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Introduction

The Australian genera *Banksia* and *Dryandra* are cultivated for cut flower production, with *B. coccinea*, the scarlet banksia, the most important. They are sister genera within the family Proteaceae, and breeding is currently underway to develop improved *Banksia* cultivars for the cut flower industry (Sedgley et al. 1994, 1996).

Banksia comprises two subgenera, *Isostylis* and *Banksia*. The former group is small and the species are superficially similar to *Dryandra*, leading George (1981) to propose that *Isostylis* may be closer to *Dryandra* than to subgenus *Banksia*. Nevertheless, based on three distinct morphological characters he placed it as a subgenus of *Banksia*, with the comment that separate generic status may be appropriate. There is speculation on the relationships within subgenus *Banksia*, and between subgenus *Isostylis*, subgenus *Banksia* and genus *Dryandra*. The position of *B. coccinea* in the genus is unclear, as it has a number of distinct features and no obvious relatives. This work aims to clarify its relationships to identify the most productive crosses for cultivar development via interspecific or intergeneric hybridisation. The research uses random amplified polymorphic DNA (RAPD) markers and non-coding chloroplast (cp) DNA sequences to determine genetic and taxonomic relationships among *Banksia* species and between *Banksia* and *Dryandra*.

Materials and methods

Plant material

Seeds of 36 species of *Banksia* and 3 of *Dryandra* were obtained from a commercial source. Fresh leaf material of *Musgravea heterophylla*

was collected from Atherton, Queensland (QRS Arboretum No. 1372). The species chosen represented three genera, *Banksia* and *Dryandra* with *Musgravea* as the outgroup, and two subgenera, two sections and 13 series within genus *Banksia* (George 1981, 1988) (Table 1). Ten seeds of each species of *Banksia* and *Dryandra* were randomly selected for RAPD analysis, and the DNA bulked to give one sample per species. For cpDNA analysis one seed per species was chosen. One leaf was selected from *M. heterophylla* for both analyses.

DNA extraction

DNA was extracted from each seed using a modified method of Weining and Langridge (1991), comprising: phenol/chloroform/isopropanol extraction for 5 min on ice, DNA precipitation for 1 min with ice-cold isopropanol and sodium acetate, with DNA recovered by centrifugation at 12 000 rpm for 10 min. The pellet was washed twice with 70% ethanol, dried and dissolved in 50 µl of TE buffer, with 1.0 µl of RNAase (R40: 40 mg/ml RNAase A in TE), and stored at 4°C for up to 1 month. DNA was extracted from fresh leaf material of *M. heterophylla* using the extraction method for seedling material of Maguire et al. (1994). DNA was subjected to gel electrophoresis on a 1.6% agarose gel in TBE buffer (Sambrook et al. 1989) and stained with ethidium bromide. DNA concentration was estimated by visual assessment of band intensities, compared to salmon sperm genomic DNA standards. The DNA content was adjusted to 10 ng μl^{-1}.

Table 1 Species included in RAPD and cpDNA analysis. Species represent three genera, *Banksia*, *Dryandra* and *Musgravea*, and two subgenera, two sections and 13 series of genus *Banksia*. Taxonomy follows George (1981, 1988)

	RAPDs	cpDNA
GENUS *BANKSIA*		
SUBGENUS *BANKSIA*		
Section *Banksia*		
Series *Salicinae*:	*B. integrifolia*	*B. integrifolia*
	B. robur	
Series *Grandes*:	*B. grandis*	
	B. solandri	
Series *Quercinae*:	*B. quercifolia*	
Series *Bauerinae*:	*B. baueri*	
Series *Banksia*:	*B. serrata*	*B. serrata*
	B. baxteri	(2 specimens)
	B. candolleana	
	B. menziesii	
Series *Crocinae*:	*B. burdettii*	
	B. prionotes	
Series *Cyrtostylis*:	*B. ashbyi*	*B. media*
	B. attenuata	
	B. audax	
	B. elderiana	
	B. laevigata	
	B. lindleyana	
	B. praemorsa	
Series *Prosratae*:	*B. blechnifolia*	
	B. repens	
Series *Tetragonae*:	*B. caleyi*	
	B. lemanniana	
Series *Coccineae*:	*B. coccinea*	*B. coccinea*
Section *Oncostylis*		
Series *Spicigerae*:	*B. ericifolia*	*B. ericifolia*
	B. occidentalis	*B. spinulosa*
	B. tricuspis	
Series *Dryandroideae*:	*B. dryandroides*	
Series *Abietinae*:	*B. meisneri*	*B. sphaerocarpa*
	B. pulchella	
SUBGENUS *ISOSTYLIS*	*B. cuneata*	*B. cuneata*
	B. ilicifolia	*B. ilicifolia*
	B. oligantha	
GENUS *DRYANDRA*	*D. formosa*	*D. formosa*
	D. polychephala	*D. polychephala*
	D. carlinoides	*D. carlinoides*
GENUS *MUSGRAVEA*	*M. heterophylla*	*M. heterophylla*

RAPD analysis

DNA amplification was performed in a MJ Research Thermal Cycler. The programme commenced with an initial denaturation step at 94°C for 5 min, followed by 40 cycles of 94°C for 1 min, 36°C for 1 min, 72°C for 2 min and terminated with a final extension step at 72°C for 5 min. Optimised reaction conditions were carried out in a 25-µl total volume containing 1 × *Taq* buffer (Gibco-BRL), 3 m*M* $MgCl_2$, 200 µ*M* of each dNTP (dGTP, dATP, dCTP, dTAP), 1 unit of *Taq* polymerase (Gibco-BRL), 0.5 µl T4 gene 32 protein (Boehringher Mannheim), 1 µ*M* 10-mer primer (Operon Technologies) and 10 ng μl^{-1} template DNA. Each reaction mix was overlaid with polymerase chain reaction (PCR)-grade paraffin oil. DNA amplification fragments were separated by 2% agarose gel (Seakem, Promega) electrophoresis using TBE buffer (Sambrook et al. 1989). A negative control was added in each run to test for contamination. In order to test reproducibility, we tested the selected primers three times on the same sample, for a random subset of three DNA samples. Interpretation of band homology between gels was simplified by placing a standard DNA sample and a DNA marker (pGEM) in each gel. Gels were stained with ethidium bromide, and fragment patterns were photographed under UV light with Polaroid 667 film for further analysis. Polaroid photographs were scanned using a transmission scanner (Hewlett Packard Scanjet IIcx/T). The intensity and molecular weight of each visible band was determined using the software CREAM TM. (Kem-En-Tec Software Systems, Blue Sky Scientific).

Sixty primers were evaluated for their suitability in a pilot survey (series OPA, OPB and OPC, Operon Technologies). Fifteen were selected (Table 2) which gave reproducible and informative markers. Bands were scored as present (1) or absent (0) for all individuals, and a matrix of RAPD phenotypes was assembled. Each individual was represented by a vector of 1 s and 0 s for the presence or absence of any particular band for all the primers used in the study.

Two methods were used to analyse the RAPD data. A genetic distance matrix (Nei and Li 1979), with group average (UPGMA) clustering was used to produce a dendrogram. The second method applied phylogenetic analysis using the parsimony programme PAUP (Swofford 1990). Genetic distance was calculated as $D = 1 - F$, where F is similarity calculated using the Nei and Li (1979) matching coefficient method [2*n11/((2*n11) + n01 + n10)], where n = number of band positions; n11 = the number of positions where x = 1, y = 1; n00 = the number of positions where x = 0, y = 0; n01 = the number of positions where x = 0 and y = 1; n10 = the number of positions where x = 1 and y = 0 and x, y = individuals being compared. The distance matrix was calculated using the statistical package, GENSTAT 5 (Payne 1987), and UPGMA clustering was performed by the PATN programme package (Belbin 1991). Phylogenetic analysis was performed using PAUP 3.1.1 (Swofford 1990). The phylogeny was assessed using the heuristic search method of PAUP (character optimisation ACCTRAN, MULPARS and TBR branch swapping options). To ensure that all islands of most parsimonious trees were found (Maddison 1991), the search was repeated 100 times with RANDOM addition and with a maximum of 100 trees saved at each replication. The resulting most parsimonious trees produced by this process were subjected to successive weighting to minimise homoplasy. The matrix was

Table 2 RAPD primer sequence and band data for each of the 15 selected primers over the complete data set of 37 taxa producing 791 bands

Primer	Sequence 5′-3′	Total bands	Polymorphic	Monomorphic	Unique to one species
OPA-1	CAGGCCCTTC	49	49	0	7
OPA-3	AGTCAGCCAC	56	56	0	7
OPA-5	AGGGGTCTTG	55	55	0	9
OPA-7	GAAACGGGTG	53	53	0	6
OPA-13	CAGCACCCAC	53	53	0	9
OPA-20	GTTGCGATCC	51	51	0	15
OPB-1	GTTTCGCTCC	52	52	0	14
OPB-3	CATCCCCCTG	54	54	0	13
OPB-4	GGACTGGAGT	50	50	0	9
OPB-6	TGCTCTGCCC	45	45	0	9
OPB-7	GGTGACGCAG	50	50	0	12
OPB-10	CTGCTGGGAC	60	60	0	8
OPB-11	GTAGACCCGT	54	54	0	11
OPB-17	AGGGAACGAG	49	49	0	11
OPC-2	GTGAGGCGTC	60	60	0	7
	Total bands	791	791	0	147
	Mean per primer	52.7	52.7	0.0	9.8

reweighted until the tree-length values stabilised. Equally short solutions were used to produce a consensus tree.

cpDNA analysis

DNA amplification was performed as for RAPD analysis except that the programme commenced with an initial step of 94°C for 2 min, followed by 35 cycles of 1 min at 94°C, 1 min at 58°C, and 2 min at 72°C and T4 gene 32 was omitted. The intergeneric spacer between the *trn*L (UAA) 3′ exon and the *trn*F (GAA) gene was amplified using the primer sequences GGTTCAAGTCCCTCTATCCC and ATTTGAACTGGTGACACGAG (Taberlet et al. 1991). Following amplification, excess primers and deoxynucleotide triphosphates were removed from samples using polyethylene glycol (PEG) precipitation in magnesium chloride (Nicoletti and Condorelli 1993). Direct PCR sequencing of the purified fragment was carried out using standard conditions in the DyeDeoxy Terminator Sequencing Kit of Applied Biosystems, then automatically sequenced on an Applied Biosystems Model 373A. Both directions of the non-coding region were sequenced, and a consensus sequence was determined for each species.

Multiple alignments of the sequences of the non-coding region were performed manually. Phylogenetic relationships within *Banksia*, and between *Banksia* and *Dryandra*, using *Musgravea* as the outgroup, were analysed using PAUP 3.1.1. (Swofford 1990). The phylogeny was assessed using the heuristic search method of PAUP (character optimisation ACCTRAN, MULPARS and TBR branch swapping options). To ensure that all islands of most parsimonious trees were found (Maddison 1991), we repeated the search 100 times with RANDOM addition and with a maximum of 100 trees saved at each replication.

Results

RAPD analysis

A total of 791 bands was scored, with an average of 52.7 bands per primer (Table 2). The number of polymorphic markers was high, with no monomorphic bands, and the number of bands unique to only 1 species comprised 18.5% of the total.

In cluster analysis of genetic distance values, *Musgravea* formed a small group with *Banksia* subgenus *Isostylis*, and the 3 species of *Dryandra* formed a small group with *B. coccinea*. For the remainder of genus *Banksia*, species pairs and closely related species generally formed groups (Fig. 1). The cluster involving *Musgravea* and subgenus *Isostylis* is interesting, as this group is closer to many *Banksia* species than they are to each other. UPGMA clustering forms groups with increasing genetic distance. The dendrogram shows approximately 7–8 main groups, linked together at genetic distance values ranging from 0.70–0.78. The higher levels of clustering between these main groups is tenuous, however clustering within these groups is likely to be real. Within the main clusters, species pairs and closely related species group together, such as *B. occidentalis* and *B. meisneri*, *D. polycephala*, *D. carlinoides* and *D. formosa*, *B. ericifolia* and *B. dryandroides*, *B. caleyi* and *B. lemanniana*, *B. grandis* and *B. solandri*, *B. audax* and *B. lindleyana*, *B. oligantha* and *B. cuneata*, and *B. ashbyi* and *B. elderiana*.

The PAUP programme identified two minimum length trees (length = 4079) with a consistency index of 0.929. Both the strict consensus tree and the majority rule tree show that species within series *Grandes* and *Tetragonae* grouped together and that closely related species between series grouped together (Fig. 2). There were close relationships between species in series *Banksia* and *Crocinae*, series *Dryandroideae*, *Spicigerae* and *Abietinae* and series *Bauerinae* and *Cyrtostylis*. *Dryandra* was polyphyletic within *Banksia*. *Dryandra polycephala* and *D. carlinoides*, as sister taxa, were part of a clade with *Banksia* species, whereas *D. formosa* was sister to *B. coccinea*.

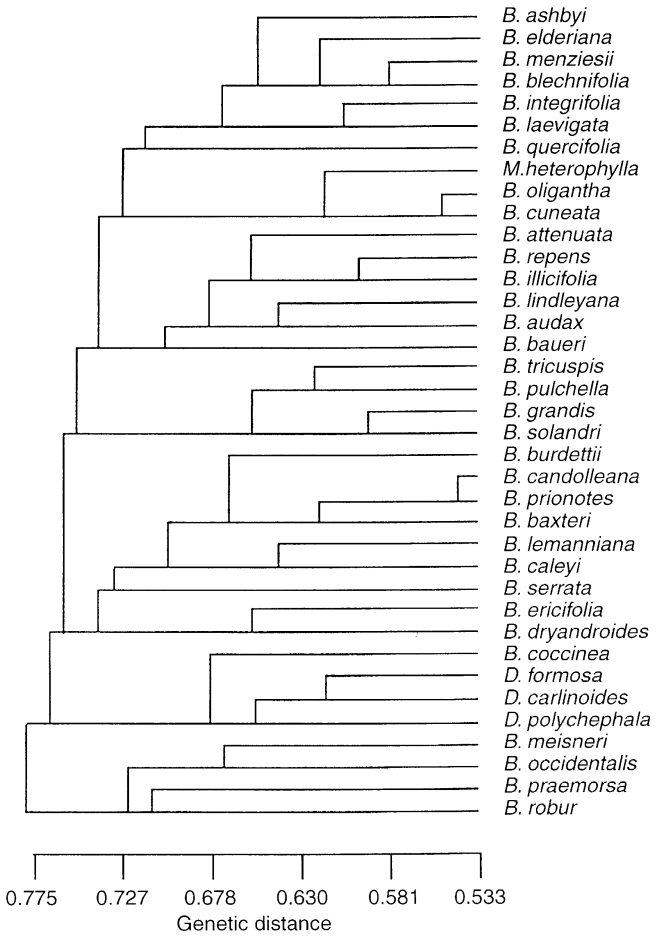

Fig. 1 Dendogram of *Banksia*, *Dryandra* and *Musgravea* species generated by cluster (UPGMA) analysis of genetic distance values generated from 791 RAPD bands using 15 primers. Genetic distance (D) was calculated as $D = 1 - F$, where F = similarity calculated using the method of Nei and Li (1979)

Chloroplast DNA analysis

Double-stranded DNA amplification products were obtained for all species. In the consensus sequence of the spacer region between the *trn*L (UAA) 3′ exon and the *trn*F (GAA) gene, the size of the aligned sequence for all species was 413 bp (Fig. 3). There was no intraspecific variation between the two individuals of *B. serrata*.

The single, most parsimonious tree found using PAUP had a consistency index of 0.983, tree length of 60, and a retention index of 0.909 (Fig. 4). Generally, most *Banksia* species fell into the two main sections of *Banksia*, subgenus *Banksia*, based on morphological characters, although *B. ericifolia*, *B. spinulosa* and *B. sphaerocarpa* of section *Oncostylis* formed a clade which included *B. integrifolia*. A second clade comprised *B. media* and the 2 individuals of *B. serrata*, representing section *Banksia*. *Banksia coccinea* and two *Dryandra* species, *D. carlinoides* and *D. polycephela*, were unresolved at the polytomy with the two sections of subgenus *Banksia*. *Banksia illicifolia*, *D. formosa* and *B. cuneata* were basal to this polytomy with the related two members of *Banksia* subgenus *Isostylis* separated.

Discussion

This study has clarified the position of *B. coccinea* within the genus and has indicated possible interspecific and intergeneric crosses which may be productive in plant breeding. RAPD and cpDNA analysis showed that *B. coccinea* is related to both the *Banksia* and *Oncostylis* sections of subgenus *Banksia*, and also has affinity with species from the sister genus *Dryandra*. All of these include species with ornamental merit which would be useful for hybridisation. In contrast, *B. coccinea* is not so closely related to subgenus *Isostylis*, and such crosses may be less productive.

The technical simplicity of the RAPD technique has facilitated its use in the analysis of genetic relationships in several genera (Wilikie et al. 1993; Demeke et al. 1992; Aboelwafa et al. 1995), but RAPDs are not generally considered to be informative at the distantly related species level. Concerns regarding RAPD-generated phylogenies at higher levels include homology of bands showing the same rate of migration, causes of variation in fragment mobility and origin of sequences in the genome (Stammers et al. 1995). In RAPD analysis, many loci are examined at the same time, which minimises the effect of loci which are under selection pressure, thereby giving divergence of the whole genome. RAPDs are appropriate for closely related groups, as shown by this study and by studies on *Lolium* (Stammers et al. 1995), *Lotus* (Campos et al. 1994), *Brassica* (Demeke et al. 1992) and *Allium* (Wilikie et al. 1993).

Chloroplast DNA has been used extensively to infer plant phylogenies at different taxonomic levels. Direct sequencing of PCR products is becoming a rapidly expanding area of plant systematics (Clegg and Zurawski 1991). The *rbc*L gene encoding the large subunit of RUBISCO has been sequenced from many plant taxa but is considered to be too conservative to resolve relationships between closely related genera (Xiang et al. 1993). Comparisons of the rates of *rbc*L and two non-coding regions of the cpDNA, the *trn*L (UAA) intron and the intergeneric spacer between the *trn*L (UAA) 3′ exon and the *trn*F (GAA) gene, have been made in several genera, and these have shown that these regions evolve faster, providing greater resolution at the generic and intrageneric level (Taberlet et al. 1991; Ferris et al. 1993; Gielly and Taberlet 1994).

Molecular analysis of *Banksia*, using RAPDs and two statistical approaches, and cpDNA sequencing gave broadly similar groupings to the classification of George (1981, 1988). It is interesting that *B. coccinea* grouped with species of *Dryandra* in all analyses,

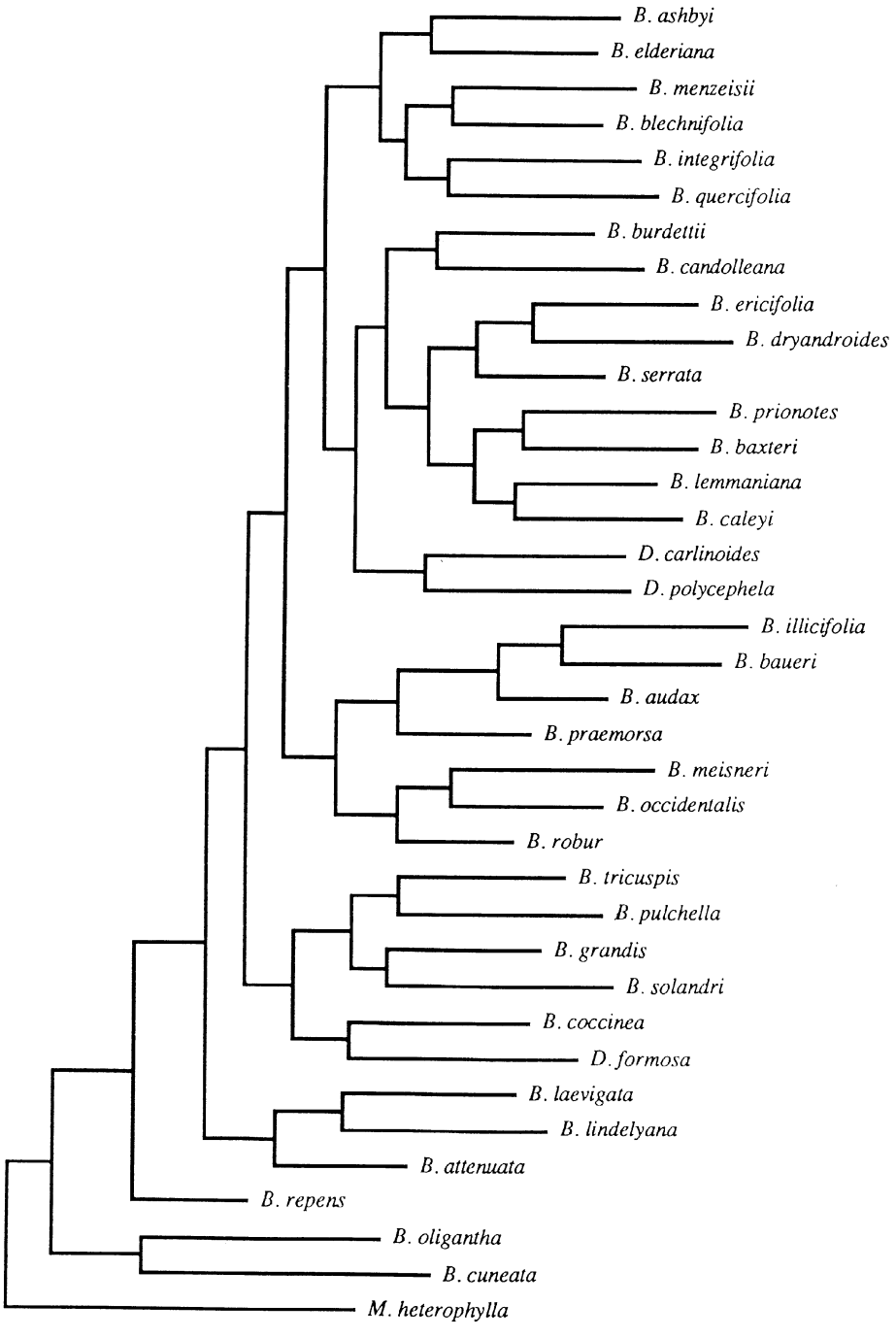

Fig. 2 Majority rule consensus tree obtained from PAUP, tree length = 4079; consistency index = 0.929. Taxa represent two closely related genera, *Banksia* and *Dryandra*, with *Musgrevea* as the outgroup in the analysis. Tree was generated from 791 RAPD bands produced with 15 primers

indicating a close relationship. In addition, *Dryandra*, in the presence of an outgroup, could not be clearly distinguished as a monophyletic clade from *Banksia*. It is generally agreed that *Banksia* and *Dryandra* are sister taxa, with parallel developments in the two genera. Few morphological characters separate the two, so it could be suggested that *Banksia* and *Dryandra* may be artificial genera. Pollen-pistil data with *B. coccinea* and *Dryandra* species also show more compatibility than some *Banksia* interspecific crosses (Maguire and Sedgley 1997). Molecular data have suggested that widely accepted views of separate genera may be artificial in other plant groups such as *Eucalyptus* and related genera (Sale et al. 1996; Ladiges et al. 1995). When RAPDs were used there were close relationships between species in series *Banksia* and *Crocinae*, series *Dryandroideae*, *Spicigerae*, and *Abietinae* and series *Bauerinae* and *Cyrtostylis*. The close relationship of series *Crocinae* and *Banksia* has been shown previously using pollen-pistil compatibility and cladistic analysis (Sedgley et al. 1994; Thiele 1993). The series *Spicigerae*, *Abietinae* and *Dryandroideae* grouped in

Fig. 3 For legend see page 259

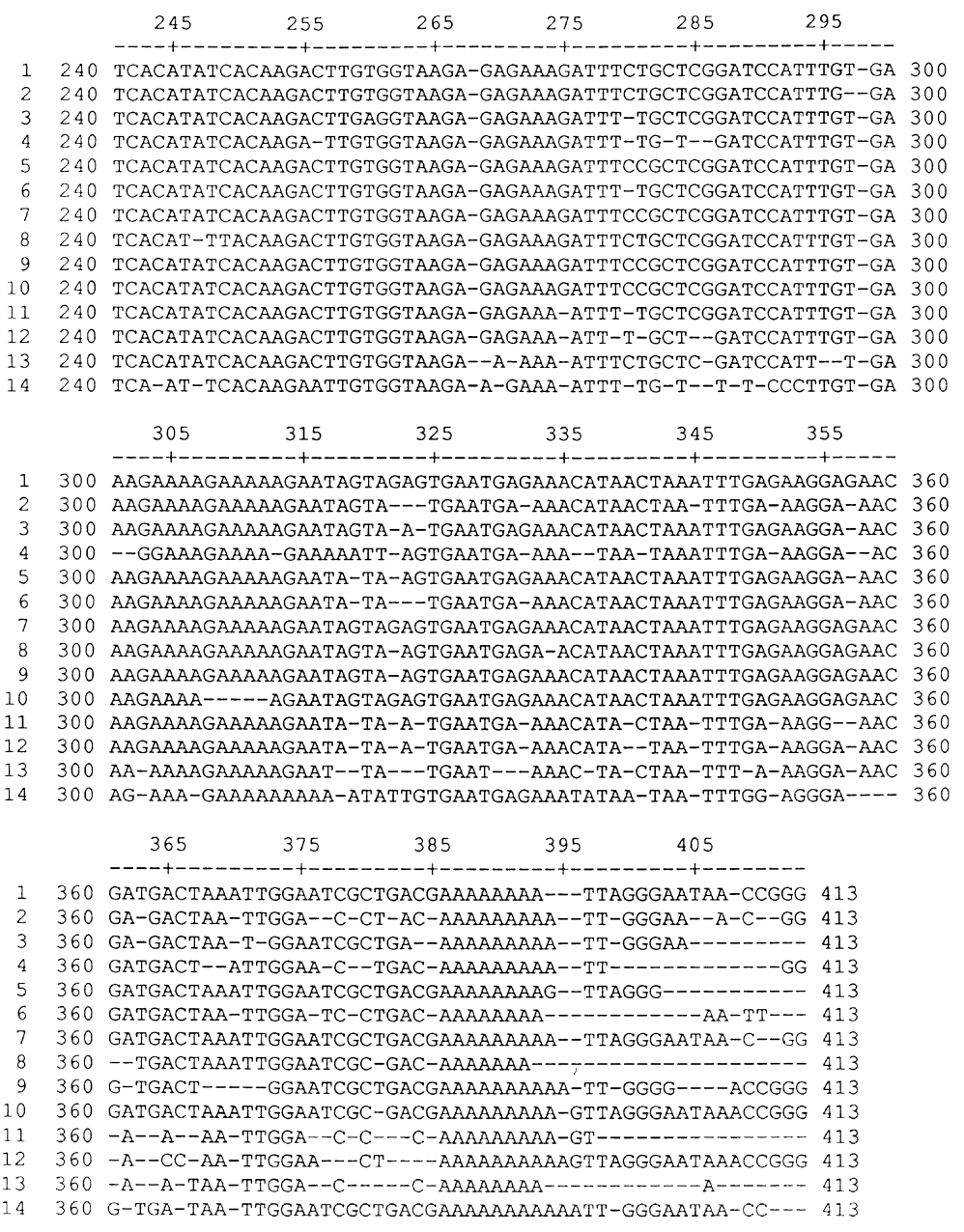

Fig. 3 Complete nucleotide sequences of the spacer between the *trn*L (UAA) and *trn*F (GAA) gene in *Banksia*, *Dryandra* and *Musgravea* (length of alignment 413 bp). *1 B. serrata* (individual 1), *2 B. serrata* (individual 2), *3 B. coccinea*, *4 B. cuneata*, *5 B. ericifolia*, *6 B. illicifolia*, *7 B. integrifolia*, *8 B. media*, *9 B. sphaerocarpa*, *10 B. spinulosa*, *11 D. carlinoides*, *12 D. formosa*, *13 D. polycephala*, *14 M. heterophylla*

section *Oncostylis*, subgenus *Banksia*, confirming the close relationship of these series (George 1981). Subgenus *Isostylis* formed a group with *Musgravea*, separate from the rest of *Banksia*, showing more genetic distinctness than subgenus *Banksia* and genus *Dryandra*. When cpDNA was used, subgenus *Isostylis* and *D. formosa* were basal to the remainder of *Banksia* and *Dryandra*. George (1981) indicated that subgenus *Isostylis* may be more closely related to *Dryandra* than *Banksia*, but placed it in *Banksia* with a note that a new genus may be appropriate.

The phylogeny obtained for *Banksia* using cpDNA sequence data, show broad species grouping into the two sections of subgenus *Banksia*, section *Banksia* and section *Oncostylis*. *Banksia coccinea* is distinct from either section, grouping instead in a polytomy with 2 species of *Dryandra* at the node with the two sections of subgenus *Banksia*, thus suggesting that a third section of the genus containing *B. coccinea* may be appropriate. This separation has already been proposed, based on morphological characters and pistil-pollen compatibility data (Maguire et al. 1996). The node between the two sections of *Banksia* with *B. coccinea* and the 2 *Dryandra* species is unresolved, and the relationship of *B. coccinea* to the *Dryandra* species is unclear. The non-coding region between *trn*L and

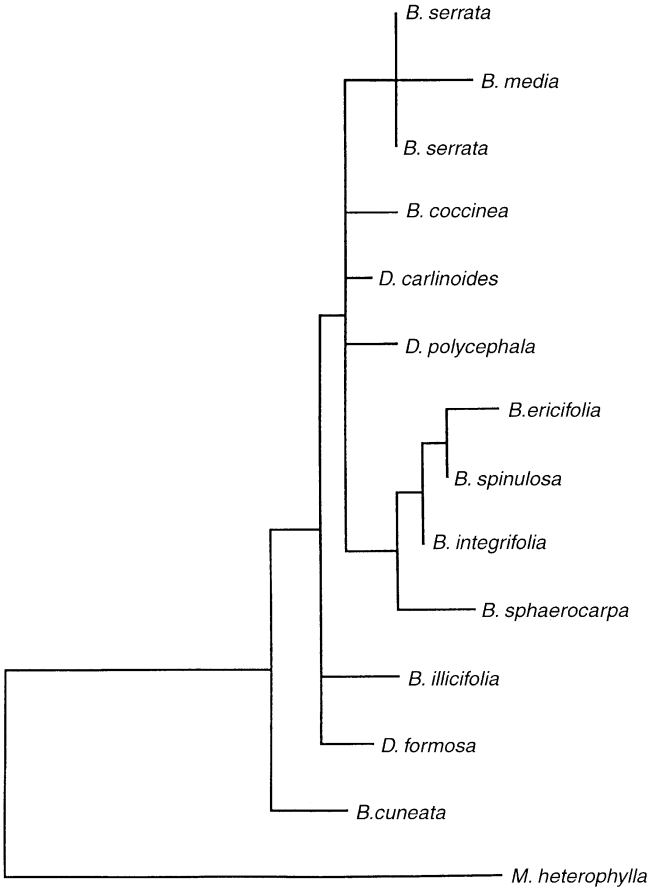

Fig. 4 Phylogenetic relationships within *Banksia* and related genera. Strict consensus of the trees was retained by the heuristic search algorithm of PAUP based on sequences of the spacer between *trn*L and *trn*F. Tree length, 60; consistency index, 0.983; retention index, 0.909

*trn*F appears to be too conservative to clearly resolve these relationships, and a faster evolving region may be more appropriate.

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