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Molecular Variation and Fingerprinting of *Leucadendron* Cultivars (Proteaceae) by ISSR Markers

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• *Background and Aims* There are more than 80 species of *Leucadendron* and most are used as cut flowers. Currently, more than 100 cultivars are used by industry and many of them are interspecific hybrids. The origin of most cultivars is unclear and their genetic diversity and relationships have not been studied. This investigation was carried out to evaluate the genetic variation and relationships among 30 *Leucadendron* cultivars.
• *Methods* ISSR markers were applied to determine the genetic variation and to discriminate *Leucadendron* cultivars. Sixty-four ISSR primers were screened and 25 primers were selected for their ability to produce clear and reproducible patterns of multiple bands.
• *Key Results* A total of 584 bands of 305–2400 bp were amplified, of which 97 % were polymorphic. A dendrogram generated using the Unweighted Pair Group Method with Arithmetic Average based on a distance measure of total character difference showed that the *Leucadendron* cultivars clustered into two main groups. Twenty-four of the 30 cultivars can be unequivocally differentiated, but identical profiles were observed for three cultivar pairs, 'Katie's Blush' and 'Silvan Red', 'Highlights' and 'Maui Sunset', and 'Yellow Crest' and 'Yellow Devil'.
• *Conclusions* ISSR profiling is a powerful method for the identification and molecular classification of *Leucadendron* cultivars. A fingerprinting key was generated based on the banding patterns produced using two ISSR primers (UBC_{856} and UBC_{857}). In addition cultivar-specific ISSR bands were obtained for 17 of the 30 *Leucadendron* cultivars tested.

Key words: Fingerprinting key, genetic variation, ISSR, *Leucadendron*, molecular relationships, Proteaceae.

INTRODUCTION

Leucadendron are Proteaceae that produce male and female flowers on separate plants. The flowers have distinct and colourful petal-like bracts (Vogts, 1982; Matthews, 2002) that, along with long stems and long-lasting foliage, make many *Leucodendron* species highly desirable cut flowers. There are 80 species of *Leucadendron*. Based on fruit characteristics, the species have been divided into two sections, *Alatosperma* and *Leucadendron*, with each section having several subsections (Williams, 1972).

There are more than 100 *Leucadendron* cultivars (International Proteaceae Register, 2002). The most widely grown cultivar, 'Safari Sunset', is grown in Australia, New Zealand, South Africa, Hawaii and Israel (Littlejohn and Robyn, 2000). Several commercial cultivars (Littlejohn *et al*., 1998; Sedgley and Yan, 2003) are the result of interspecific hybridization (Van den Berg and Brits, 1990; Littlejohn *et al*., 1995; Yan *et al*., 2001*a*, *b*).

There is limited knowledge regarding the genetic diversity and interspecies relationships in *Leucadendron*, limiting the efficiency of breeding programmes. Moreover, the parentage of *Leucadendron* cultivars is not always certain due to inadequate documentation. Some cultivars may have arisen from selection of seed populations, while others have arisen from hybridization of undocumented parents (Littlejohn and Robyn, 2000). Therefore, methods for the fast and accurate identification of *Leucadendron* species and cultivars would be of substantial benefit to the *Leucadendron* cut-flower and breeding industry.

Random amplified polymorphic DNA (RAPD) markers (Williams *et al*., 1990) and ISSR markers (Zietkiewicz *et al*., 1994) are two molecular typing approaches that have been used to detect variation among plants. Each method has been used extensively to identify and determine relationships at the species and cultivar levels (Rajaseger *et al*., 1997; Raina *et al*., 2001; Martins *et al*., 2003). These methods are widely applicable because they are rapid, inexpensive, simple to perform, do not require prior knowledge of DNA sequence and require very little starting DNA template (Esselman *et al*., 1999).

The ISSR method has been reported to produce more complex marker patterns than the RAPD approach (Parsons *et al*., 1997; Chowdhury *et al*., 2002), which is advantageous when differentiating closely related cultivars. In addition, ISSR markers are more reproducible than RAPD markers (Goulão and Oliveira, 2001), because ISSR primers, designed to anneal to a microsatellite sequence, are longer than RAPD primers, allowing higher annealing temperatures to be used. ISSR analysis has been used for cultivar identification in numerous plant species, including rice (Joshi *et al*., 2000), apple (Goulão and Oliveira, 2001) and strawberry (Arnau *et al*., 2003).

This paper reports on the use of ISSR markers to differentiate *Leucadendron* cultivars, to determine the molecular relationships among the cultivars tested and to develop a fingerprinting key for *Leucadendron*.

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TABLE 1. Leucadendron *cultivars used in this study*

Cultivars	Parental origin	Source of collection	References
Buttercup	*Leucadendron* sp.	Amarilo Proteas[§]	IPR*
Corringle Gold	*L. gandogeri* Schinz ex Gand. × *L. spissifolium* (Salisb. ex Knight) I.Williams	Proteaflora	IPR, Matthews (2002)
Devil Blush	*L. salignum* Berg.	Proteaflora	Proteaflora[†]
Fire Glow	*L. salignum* Berg.	Proteaflora	IPR, Proteaflora
Gem	*L. laureolum* (Lam.) Fourc. × *L. salignum* Berg.	Proteaflora	Matthews (2002)
Highlights	*L. laureolum* (Lam.) Fourc. × *L. salignum* Berg.	Proteaflora	IPR
Inca Gold	*L. laureolum* (Lam.) Fourc. × *L. salignum* Berg. (yellow form)	Amarilo Proteas	Matthews (2002)
Jubilee Crown	*L. laxum* I.Williams hybrid	Amarilo Proteas	IPR, Littlejohn and Robyn (2000)
Katie's Blush	*L. laureolum* (Lam.) Fourc. × *L. salignum* Berg.	Amarilo Proteas	Proteaflora
Maui Sunset	*L. laureolum* (Lam.) Fourc. × *L. salignum* Berg.	Amarilo Proteas	IPR, Matthews (2002)
Pisa	*L. floridum* R.Br hybrid	Amarilo Proteas	IPR, Matthews (2002)
Pixie Red	*L. salicifolium* (Salisb.) I.Williams × *L. procerum* (Salisb. ex Knight) I.Williams	UWA	Sedgley and Yan (2003)
Pom Pom	*L. discolor* Buex ex Meisn.	Proteaflora	IPR
Red Devil	*L. salignum* Berg.	Proteaflora	IPR, Proteaflora
Red Centre	*L. discolor* Buex ex Meisn.	Proteaflora	Proteaflora
Safari Sunset	*L. laureolum* (Lam.) Fourc. × *L. salignum* Berg.	Amarilo Proteas	Matthews (2002)
Safari Goldstrike	*L. strobilinum* Druce × *L. laureolum* (Lam.) Fourc.	Proteaflora	Matthews (2002)
Silvan Red	*L. laureolum* (Lam.) Fourc. × *L. salignum* Berg.	Proteaflora	IPR, Matthews (2002)
Spring Gold	*L. gandogeri* Schinz ex Gand.	Proteaflora	Proteaflora
Summer Sun	*L. laureolum* (Lam.) Fourc. × *L. discolor* Buex ex Meisn.	Proteaflora	Proteaflora
Tall Red	*L. salignum* Berg. × *L. eucalyptifolium* Buex ex Meisn.	Proteaflora	Proteaflora
Yellow Devil	*L. salignum* Berg.	Proteaflora	IPR, Proteaflora
Yellow Crest	*L. salignum* Berg.	Amarilo Proteas	IPR
436	*L. coniferum* Meisn. × *L. discolor* Buex ex Meisn.	UWA	UWA[‡]
772	*L. floridum* R.Br × *L. discolor* Buex ex Meisn.	UWA	UWA
802	*L. floridum* R.Br × *L. gandogeri* Schinz ex Gand.	UWA	UWA
868	*L. floridum* R.Br × *L. procerum* (Salisb. ex Knight) I.Williams	UWA	UWA
1386	*L. uliginosum* R.Br × *L. discolor* Buex ex Meisn.	UWA	UWA
1325	*L. strobilinum* Druce × *L. procerum* (Salisb. ex Knight) I.Williams	UWA	UWA
1424	*L. uliginosum* R.Br × *L. salicifolium* (Salisb.) I.Williams	UWA	UWA

*International Proteaceae Register (http://www.nda.agric.za/docs/Protea2002/proteaceae_register.htm).
[†]Proteaflora (http://www.proteaflora.com/).
[‡]G. Yan, R. Sedgley and B. Croxford (unpubl. res.).
[§]Hilltop Rise, Karnup, WA 6176.

MATERIALS AND METHODS

Plant materials

Leaf tissue for DNA extraction was collected from the 30 *Leucadendron* cultivars listed in Table 1.

DNA extraction and electrophoresis

Genomic DNA was extracted using a DNeasy Plant Mini Kit (Qiagen, Clifton Hill, Victoria, Australia). Approximately 0·1 g of leaf material from one individual plant for each cultivar was ground to a fine powder in liquid nitrogen with a mortar and pestle before isolation of DNA according to the manufacturer's instructions. DNA was visualized by agarose gel electrophoresis followed by staining with ethidium bromide (Sambrook *et al*., 1989). Known amounts of lambda DNA (MBI, Fermentas, Hanover, MD, USA) were included on the gel to quantify the DNA.

ISSR amplification

ISSR primers (UBC set 9) were from the Biotechnology Laboratory, The University of British Columbia, Canada (Table 2). Optimal conditions for DNA amplification were empirically determined by testing different concentrations of genomic DNA (10, 15, 25 and 40 ng), $MgCl_2$ (1·5, 2, 2·5, 3·0 mM) and primers (0·15, 0·2, 0·3 and 0·4 μM). The optimal annealing temperature was found to vary according to the base composition of the primers (Table 2). PCR amplifications were performed in 25-μL reaction mixtures containing 10 ng DNA, 1·5 mM $MgCl_2$, 1× PCR buffer (10 mM KCl, 10 mM $(NH_4)_2SO_4$, 20 mM Tris–HCl, 2 mm $MgSO_4$, 0·1 % Triton X-100 pH 8·8), 0·3 μM primer, 200 μM of each dNTP (Promega, Annandale, NSW, Australia) and 1 unit of Taq DNA polymerase (New England BioLabs, Baverly, MA, USA). To reduce background amplification, 2 % (v/v) formamide was added to the reactions (Fang and Roose, 1997; Raina *et al*., 2001). Amplifications were carried out using a thermocycler (iCycler, Biorad, Regents Park, NSW, Australia) with an initial denaturation/activation step of 4 min at 95°C, followed by 45 cycles of 30 s at 94°C, 45 s at annealing temperature (Table 2) and 2 min extension at 72°C. A final extension for 10 min at 72°C was included. Optimal conditions were determined based on the resolvable PCR products generated by each primer. A negative control which contained all PCR components except DNA (replaced by water) was included in every experiment to test for DNA contamination of the reagents.

TABLE 2. *ISSR primers used in this study and summary of ISSR markers from 30* Leucadendron *cultivars*

Primer (UBC)	Primer sequence*	Annealing temperature (°C)†	Fragment size range	Fraction polymorphic fragments‡
813	$(CT)_8T$	50	430–1625	12/12
814	$(CT)_8A$	50	385–1980	19/19
815	$(CT)_8G$	52	395–1690	27/27
817	$(CA)_8A$	52	595–1355	14/15
820	$(GT)_8T$	52	575–2055	16/16
824	$(TC)_8G$	50	595–2230	24/24
826	$(AC)_8C$	52	500–1735	23/25
834	$(AG)_8YT$	52	370–1490	34/34
836	$(AG)_8YA$	52	325–1990	30/31
840	$(GA)_8YT$	54	255–1645	35/35
843	$(CT)_8RA$	54	350–1965	21/21
845	$(CT)_8RG$	54	305–2100	18/18
848	$(CA)_8RG$	55	450–1665	18/19
852	$(CT)_8RA$	52	310–2195	17/18
855	$(AC)_8YT$	55	310–1825	27/28
856	$(AC)_8YA$	55	480–1710	14/14
857	$(AC)_8YG$	54	250–2000	29/29
858	$(TG)_8RT$	52	330–1845	21/21
859	$(TG)_8RC$	55	300–1340	25/26
860	$(TG)_8RA$	52	330–2400	17/18
889	$DBD(AC)_7$	52	320–1800	26/27
890	$VHV(GT)_7$	54	310–1575	36/37
891	$HVH(TG)_7$	54	340–1495	27/27
868	$(GAA)_6$	50	470–1675	30/30
873	$(GACA)_4$	50	595–1355	10/13
				570/584

*Single letter abbreviations for mixed-base positions: Y = (C, T), R = (A, G), B = (non A), D = (non C), V = (non T), H = (non G).
†Determined empirically.
‡Number of polymorphic fragments/number of fragments amplified.

PCR products were visualized using agarose gel electrophoresis stained with ethidium bromide (Sambrook *et al*., 1989).

Data analysis

Gels were photographed (Kodak Digital Science1D™, Eastman Kodak Co., Rochester, NY, USA) and the sizes of the fragments estimated (Digital Science 1D Image Analysis Software, Eastman Kodak Company, Rochester, USA). Each ISSR band was considered as a character and the presence or absence of the band was scored in binary code (present = 1, absent = 0). A data matrix was assembled and analysed using Phylogenetic Analysis Using Parsimony (PAUP; Swofford, 1998) and a pairwise distance matrix was generated based on total character differences. The genetic relatedness among the *Leucadendron* cultivars was analysed using Unweighted Pair Group Method with Arithmetic Average (UPGMA) based on distance measure of total character difference. Bootstrap analysis using UPGMA search with 1000 replicates was performed to obtain the confidence of branches of the tree.

RESULTS

Selection of primers and reproducibility

Initially, 64 ISSR primers were screened against genomic DNA from three *Leucadendron* cultivars ('Katie's Blush', 'Gem' and 'Red Centre') for their ability to amplify DNA fragments. Of the 64 primers, eight produced no distinct bands on a smeary background and 31 resulted in very faint bands upon a highly smeared background. The remaining 25 primers (Table 2) produced robust amplification patterns. As an example, the pattern obtained for each cultivar with primer UBC_{857} is shown in Fig. 1. Within the set of 25 primers giving robust patterns, there were 23 di-nucleotide repeat primers, 20 primers with 3′ anchors and 3 primers with 5′ anchors (Table 2). The single tri- and tetra-nucleotide repeat primers were both unanchored.

The reproducibility of the ISSR amplifications was assessed using selected primers (UBC_{820}, UBC_{826}, UBC_{834}, UBC_{860}, UBC_{868}, UBC_{890} and UBC_{891}) with different DNA samples isolated independently from the same cultivar and amplified at different times. Under the optimized PCR conditions, the banding profiles were consistent among PCR experiments (data not shown).

ISSR diversity

The set of 25 ISSR primers showed multiband patterns in each cultivar and no band was detected in any negative control amplification. This primer set amplified a total of 584 bands from the 30 *Leucadendron* cultivars tested. Primer UBC_{813} resulted in the smallest number of bands (12) and primer UBC_{890} generated the largest number of bands (37). The average number of bands per primer was 23·4. Band size ranged from 305 bp (UBC_{845}) to 2·4 kb (UBC_{860}). Among the 30 *Leucadendron* cultivars, 570 (97·6 %) of the ISSR bands were polymorphic. The percentage of polymorphic fragments per primer was 76–100 % (Table 2).

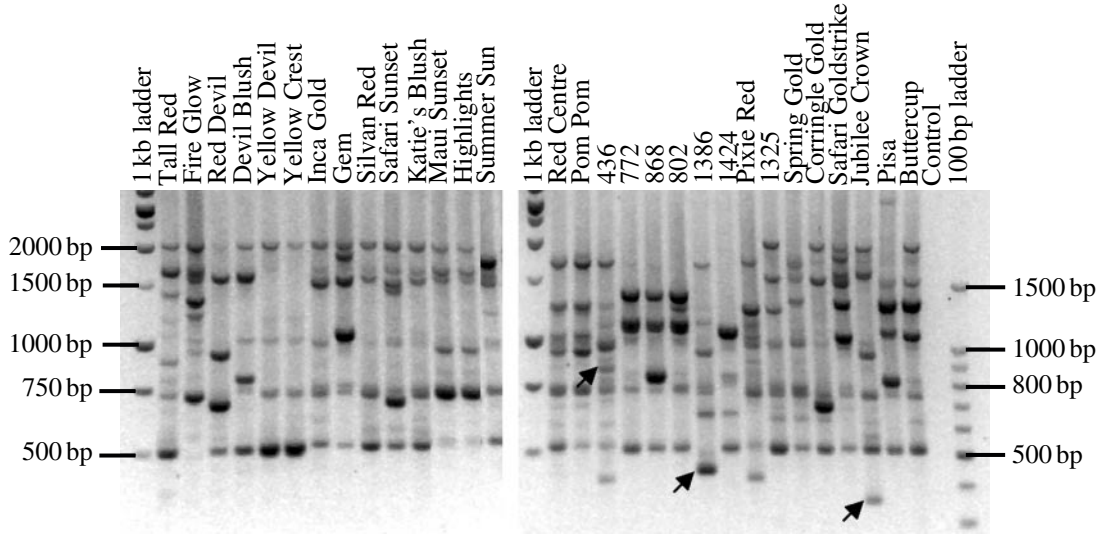

FIG. 1. ISSR marker profiles obtained from *Leucadendron* cultivars. ISSR amplification products were produced using primer UBC_{857} and separated on 1·8 % agarose gel. The cultivars (Table 1) are shown across the top of the figure. The lanes containing 1-kb and 100-bp marker ladders (Promega, Annandale, NSW, Australia) are indicated, as are the sizes of selected marker bands. The bands marked with arrows are the cultivar-specific bands amplified (Table 3).

Identical ISSR profiles were obtained for three cultivar pairs: 'Katie's Blush' and 'Silvan Red', 'Highlight' and 'Maui Sunset', and 'Yellow Crest' and 'Yellow Devil'.

Molecular relationship and fingerprinting of Leucadendron *cultivars*

UPGMA analysis based on total ISSR character difference was carried out to group the 30 *Leucadendron* cultivars. A dendrogram resulting from a cluster analysis of the distance matrix showed two main groups, designated A and B (Fig. 2). The UPGMA dendrogram showed a high confidence level. Bootstrap analysis using 1000 replicates showed that seven forks had 100 % bootstrap support and 21 of the 28 forks had greater than 50 % bootstrap support.

Cultivar-specific ISSR bands were obtained for 17 of the 30 *Leucadendron* cultivars tested (Table 3). Using the ISSR data from primers UBC_{856} and UBC_{857}, a fingerprinting key was generated (Fig. 3) that is able to distinguish 27 cultivars out of 30 cultivars.

TABLE 3. *Cultivar-specific bands revealed by ISSR fingerprinting for 17* Leucadendron *cultivars (other cultivars listed in Table 1 cannot be characterized by the presence and/or absence of specific bands)*

Cultivar	Characterized by the presence of ISSR markers*	Characterized by the absence of ISSR markers
Buttercup	$UBC_{860–2460}$	
Devil Blush	$UBC_{840–420}$, $UBC_{855–1420}$	
Fire Glow	$UBC_{855–465}$	
Gem	$UBC_{868–795}$	
Inca Gold	$UBC_{855–920}$	
Jubilee Crown	$UBC_{836–325}$, $UBC_{845–305}$, $UBC_{856–800}$, $UBC_{857–350}$	
Maui Sunset, Highlights	$UBC_{815–1685}$	
Pixie Red	$UBC_{855–1000}$, $UBC_{891–500}$	
Red Devil	$UBC_{814–805}$	
Safari Goldstrike	$UBC_{868–470}$	
Safari Sunset	$UBC_{836–590}$	
Spring Gold	$UBC_{848–1820}$	$UBC_{855–540}$
Summer Sun	$UBC_{859–825}$	
Tall Red	$UBC_{824–1260}$, $UBC_{852–310}$, $UBC_{860–1300}$	
436	$UBC_{857–870}$	
1386	$UBC_{857–450}$	
1424	$UBC_{891–525}$	$UBC_{891–670}$

*An ISSR primer (i.e. UBC_{824}) is followed by the size, in bp, of the cultivar-specific fragments.

DISCUSSION

ISSR markers

The 64 primers, including di-, tri- and tetra- nucleotide repeat primers, tested in this study amplified DNA fragments from *Leucadendron* genomic DNA with different efficiencies. Several studies have reported that 5′-anchored ISSR primers generated informative bands (Charters *et al*., 1996; Matthews *et al*., 1999). However, in the present study, primers with 3′-anchors were more successful in amplifying specific bands than primers with 5′-anchors. Most non-anchored or 5′-anchored primers produced smeared patterns, with or without faint bands. Since primer specificity determinants are located within the first eight nucleotides at the 3′ end (Caetano-Anollés, 1994), anchoring primers at their 3′ ends will lower the number of sequences which have homology to the primers, thus producing distinct bands (Parsons *et al*., 1997). The success in amplifying specific bands also depends on the anchoring motif. For example, while primers $(AG)_8T$, $(AG)_8C$ and $(AG)_8G$ produced

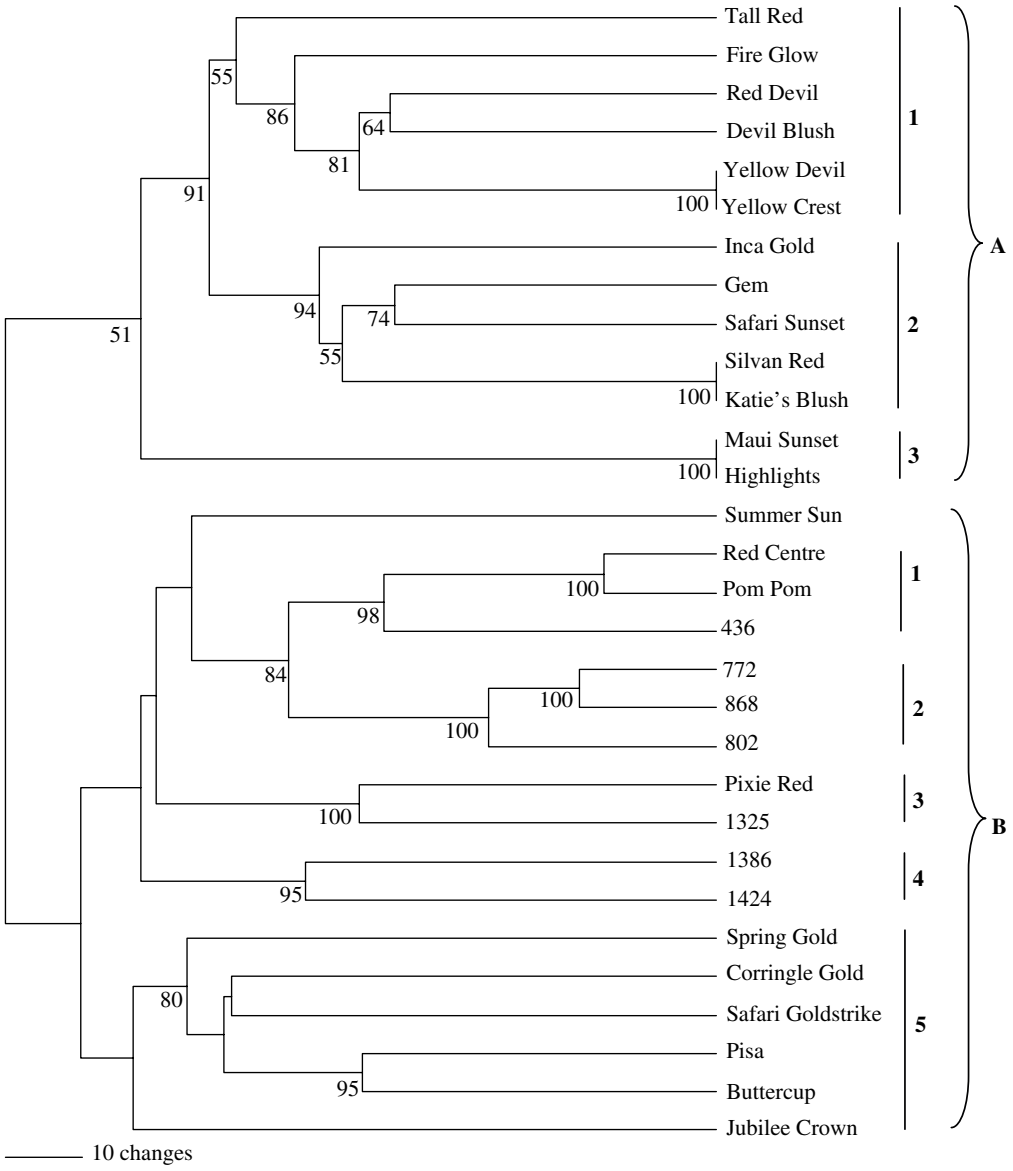

FIG. 2. Dendrogram of 30 *Leucadendron* cultivars based on UPGMA analysis of ISSR polymorphisms. The *Leucadendron* cultivars (Table 1) clustered into groups (A and B) and subgroups (numbers on left). Numbers below the lines indicate bootstrap values (percentage of 1000 replicates). Bootstrap values greater than 50 % are shown.

smeared profiles, the AG repeat primers anchored with YT or YA produced clear and distinct products.

The failure of several primers to give clear banding patterns may be because those primers require special amplification conditions, such as alternative chemical stabilizers or different annealing temperatures. The type of gel electrophoresis and staining method used can also influence the number of scorable bands and the level of polymorphism observed (Godwin *et al*., 1997, Wiesner and Wiesnerová, 2003). Both Charters *et al*. (1996) and Matthews *et al*. (1999) used polyacrylamide gels as a resolving medium and silver staining for visualization. Compared with agarose gels, polyacrylamide gels visualized by autoradiography of radiolabelled samples, or to some extent visualized by silver staining, were reported to give higher resolution (Godwin *et al*., 1997).

Fingerprinting of Leucadendron *cultivars and cultivar-specific markers*

Until now, identification of *Leucadendron* species has relied on morphological characters, especially of the fruit and seed. A fingerprinting key, based on the banding patterns of ISSR markers that can be used to identify *Leucadendron* cultivars, has now been generated (Fig. 3). The high level of polymorphism of the ISSR markers detected in *Leucadendron* facilitated the development of the DNA fingerprinting key. In fact, the fingerprinting key for *Leucadendron* cultivars could be developed using only two primers, UBC_{856} and UBC_{857} (Fig. 3). The use of a small number of primers is advantageous for reducing the time and cost of analysis. The ideal of using a single ISSR primer has been achieved for the differentiation of 30 strawberry

1. $UBC_{856–480}$ P Fire Glow, Inca Gold, Maui Sunset, Highlights, Summer Sun, Red Centre, Pom Pom, 436, 772, 868, 802, 1386, Pixie Red, 1325
   - 1.1. $UBC_{857–420}$ P 436, Pixie Red
     - 1.1.1. $UBC_{856–710}$ P **436**
     - 1.1.2. $UBC_{856–710}$ A **Pixie Red**
   - 1.2. $UBC_{857–420}$ A Fire Glow, Inca Gold, Maui Sunset, Highlights, Summer Sun, Red Centre, Pom Pom, 722, 868, 802, 1386, 1325
     - 1.2.1. $UBC_{857–1160}$ P 722, 868, 802
       - 1.2.1.1. $UBC_{857–825}$ P **868**
       - 1.2.1.2. $UBC_{857–825}$ A 722,802
         - 1.2.1.2.1. $UBC_{857–1300}$ P **802**
         - 1.2.1.2.2. $UBC_{857–1300}$ A **722**
     - 1.2.2. $UBC_{857–1160}$ A Fire Glow, Inca Gold, Maui Sunset, Highlights, Summer Sun, Red Centre, Pom Pom, 1386, 1325
       - 1.2.2.1. $UBC_{857–1300}$ P Fire Glow, Red Centre, Pom Pom, 1325
         - 1.2.2.1.1. $UBC_{857–975}$ P Fire Glow, Red Centre, Pom Pom
           - 1.2.2.1.1.1. $UBC_{857–1400}$ P **Fire Glow**
           - 1.2.2.1.1.2. $UBC_{857–1400}$ A Red Centre, Pom Pom
             - 1.2.2.1.1.2.1. $UBC_{856–1415}$ P **Pom Pom**
             - 1.2.2.1.1.2.2. $UBC_{856–1415}$ A **Red Centre**
         - 1.2.2.1.2. $UBC_{857–975}$ A **1325**
       - 1.2.2.2. $UBC_{857–1300}$ A Inca Gold, Maui Sunset, Highlights, Summer Sun, 1386
         - 1.2.2.2.1. $UBC_{857–450}$ P **1386**
         - 1.2.2.2.2. $UBC_{857–450}$ A Inca Gold, Maui Sunset, Highlights, Summer Sun
           - 1.2.2.2.2.1. $UBC_{857–1760}$ P **Summer Sun**
           - 1.2.2.2.2.2. $UBC_{857–1760}$ A Inca Gold, Maui Sunset, Highlights
             - 1.2.2.2.2.2.1. $UBC_{857–900}$ P Maui Sunsets, Highlights
             - 1.2.2.2.2.2.2. $UBC_{856–900}$ A **Inca Gold**

2. $UBC_{856–480}$ A Tall Red, Red Devil, Devil Blush, Yellow Devil, Yellow Crest, Gem, Silvan Red, Safari Sunset, Katie's Blush, 1424, Spring Gold, Corringle Gold, Safari Goldstrike, Jubilee Crown, Pisa, Buttercup
   - 2.1. $UBC_{856–780}$ P Tall Red, Red Devil, Devil Blush, Yellow Devil, Yellow Crest, Gem, Silvan Red, Safari Sunset, Katies Blush
     - 2.1.1. $UBC_{857–915}$ P **Tall Red**
     - 2.1.2. $UBC_{857–915}$ A Red Devil, Devil Blush, Yellow Devil, Yellow Crest, Gem, Silvan Red, Safari Sunset, Katie's Blush
       - 2.1.2.1. $UBC_{857–975}$ P **Red Devil**
       - 2.1.2.2. $UBC_{857–975}$ A Devil Blush, Yellow Devil, Yellow Crest, Gem, Silvan Red, Safari Sunset, Katie's Blush
         - 2.1.2.2.1. $UBC_{857–825}$ P **Devil Blush**
         - 2.1.2.2.2. $UBC_{857–825}$ A Yellow Devil, Yellow Crest, Gem, Silvan Red, Safari Sunset, Katie's Blush
           - 2.1.2.2.2.1. $UBC_{856–1310}$ P Gem, Silvan Red, Katie's Blush
             - 2.1.2.2.2.1.1. $UBC_{857–1100}$ P **Gem**
             - 2.1.2.2.2.1.2. $UBC_{857–1100}$ A Silvan Red, Katie's Blush
           - 2.1.2.2.2.2. $UBC_{856–1310}$ A Yellow Devil, Yellow Crest, Safari Sunset
             - 2.1.2.2.2.2.1. $UBC_{857–1490}$ P **Safari Sunset**
             - 2.1.2.2.2.2.2. $UBC_{857–1490}$ A Yellow Devil, Yellow Crest
   - 2.2. $UBC_{856–780}$ A 1424, Spring Gold, Corringle Gold, Safari Goldstrike, Jubilee Crown, Pisa, Buttercup
     - 2.2.1. $UBC_{857–350}$ P **Jubilee Crown**
     - 2.2.2. $UBC_{857–350}$ A 1424, Spring Gold, Corringle Gold, Safari Goldstrike, Pisa, Buttercup
       - 2.2.2.1. $UBC_{856–1310}$ P Spring Gold, Corringle Gold, Buttercup
         - 2.2.1.1. $UBC_{857–680}$ P **Corringle Gold**
         - 2.2.1.2. $UBC_{857–680}$ A Spring Gold, Buttercup
           - 2.2.1.2.1. $UBC_{857–2000}$ P **Buttercup**
           - 2.2.1.2.2. $UBC_{857–2000}$ A **Spring Gold**
       - 2.2.2.2. $UBC_{856–1310}$ A 1424, Safari Goldstrike, Pisa
         - 2.2.2.2.1. $UBC_{857–650}$ P **1424**
         - 2.2.2.2.2. $UBC_{857–650}$ A Safari Goldstrike, Pisa
           - 2.2.2.2.2.1. $UBC_{857–825}$ P **Pisa**
           - 2.2.2.2.2.2. $UBC_{857–825}$ A **Safari Goldstrike**

FIG. 3. ISSR marker fingerprinting key for *Leucadendron* cultivars generated from amplification products of primers UBC_{856} and UBC_{857}. ISSR bands are designated by the primer name (i.e. UBC_{856}) followed by the fragment size, in bp. The presence (P) or absence (A) of bands allows 24 of the *Leucadendron* cultivars (Table 1) tested to be uniquely identified (bold text).

cultivars (Arnau *et al*., 2003). The ISSR assay also identified cultivar-specific markers (Table 3) that have the potential of being used as diagnostic tools for cultivar identification, or that could be developed into cultivar-specific Sequence Characterized Amplified Region (SCAR) markers.

The *Leucadendron* fingerprinting key cannot differentiate the cultivar pairs 'Highlights' and 'Maui Sunset', 'Katie's Blush' and 'Silvan Red', and 'Yellow Devil' and 'Yellow Crest'. The International Proteaceae Register (2002) lists 'Highlight' and 'Maui Sunset' as the same cultivar. The present findings certainly indicate that these two cultivars are closely related, but a more extensive analysis is required to assert with statistical certainty that they are identical. The International Proteaceae Register (2002) lists 'Yellow Crest' and 'Yellow Devil' as separate cultivars released by different companies, while some horticulturalists believe that 'Yellow Crest' is a former name of 'Yellow Devil' (P. Armitage, pers. comm.). The present results support the view that these cultivar names are synonyms. The ISSR analysis also failed to differentiate 'Katie's Blush' and 'Silvan Red'. 'Katie's Blush' is a stable sport of 'Silvan Red' with variegated foliage (Matthews, 2002).

Relationships among Leucadendron *cultivars*

The dendrogram displaying the molecular relationships among the 30 *Leucadendron* cultivars tested separates them into two main groups. Cultivars in group A are either selections of *L. salignum* or *L. salignum* × *L. laureolum* hybrids (Matthews, 2002). Group B contains of a heterogeneous group of cultivars, but clustering is based on parental lineage. For example, the *L. discolor* cultivars 'Red Centre' and 'Pom Pom' group together with '436' which has *L. discolor* as the male parent (Fig. 2).

Group A can be divided into three clear subgroups (A1, A2 and A3), with 'Maui Sunset' and 'Highlights' being distantly separated from the other members of group A (Fig. 2). Cultivars of *L. salignum* (subgroup A1) formed a sister group to the hybrid progeny of a *L. salignum* × *L. laureolum* cross (subgroup A2). 'Tall Red' which clustered with the *L. salignum* cultivars, is recorded as a *L. salignum* × *L. eucalyptifolium* hybrid (Proteaflora, 2004). The ISSR analysis showed that 'Tall Red' is the most distantly related cultivar in the *L. salignum* branch, supporting the view that this cultivar is a hybrid.

'Maui Sunset' is recorded as the result of a cross between *L. laureolum* × *L. salignum* (International Proteaceae Register, 2002; Matthews, 2002). However, plant morphology suggests that 'Maui Sunset' is actually a trihybrid of (*L. laureolum* × *L. discolor*) × *L. salignum* (B. Croxford, The University of Western Australia, pers. comm.). 'Maui Sunset' is morphologically very similar to hybrids between *L. discolor* and *L. salignum*, but has slightly longer bracts. In addition, at one growth stage, 'Maui Sunset'/'Highlights' shows colour which is not displayed by *L. discolor* × *L. salignum* hybrids. The placement of 'Maui Sunset' on a separate branch from *L. salignum* and from the progeny of crosses between *L. salignum* and *L. laureolum* (Fig. 2) supports the view that 'Maui Sunset' is genetically dissimilar to *L. laureolum* × *L. salignum* hybrids and may be a trihybrid. Crossing *L. laureolum* × *L. discolor* results in plants that are morphologically similar to *L. laureolum*, which may explain the confusion in the parentage of 'Maui Sunset' (B. Croxford, University of Western Australia, pers. comm.).

In general, *Leucadendron* cultivars in group B cluster according to their reported pedigrees (Fig. 2 and Table 1). An exception is subgroup B5, where the reported parentage of the cultivars is broad. However, there is at least some uncertainty in the parentage of several of these cultivars. For example, 'Pisa' is only reported to be a *L. floridum* hybrid (Matthews, 2002), 'Jubilee Crown' has been only noted as a *L. laxum* hybrid (Littlejohn and Robyn 2000) and there is no clear information on the origin of 'Buttercup' (International Proteaceae Register, 2002).

This study further demonstrates that ISSR markers are a powerful tool for generating fingerprinting keys and have the potential to identify cultivar-specific markers for *Leucadendron*. The elucidation of the relationships among the 30 *Leucadendron* cultivars, the identification of species-specific ISSR markers and the generation of a fingerprinting key are important resources for the breeding and management of *Leucadendron* germplasm.

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