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The conservation of mitochondrial genome sequence in *Leucadendron* (Proteaceae)

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ABSTRACT

Pharmawati M, Yan G, Finnegan PM. 2012. The conservation of mitochondrial genome sequence in Leucadendron *(Proteaceae). Biodiversitas 13: 53-58.* Mitochondrial DNA (mtDNA) is useful for developing molecular markers and for studying plant phylogeny. However, its usefulness depends on the degree of detectable sequence variation. In seven species of the genus *Leucadendron*, PCR-RFLP failed to reveal any polymorphisms in seven separate regions of the mtDNA. Sixty-two primer pair - enzyme combinations were used to assay at least 248 restriction sites, resulting in the direct sampling of a minimum of 992 bp across 17,500 bp of mt DNA. The highly conserved nature of the mtDNA sequence in the genus *Leucadendron* was confirmed by the absence of sequence variation in the 1434 bp mtDNA *nad*1/B-C intron across these species. Mitochondrial DNA sequences are more highly conserved than the chloroplast DNA sequences in *Leucadendron* and the mtDNA sequences in many other plant genera. Phylogenetic analysis using this intron sequence was consistent with other phylogenetic analyses in regard to the position of Proteaceae.

Key words: *Leucadendron*, mtDNA, *nad1*/B-C, PCR-RFLP

INTRODUCTION

Leucadendron is a genus of South African Proteaceae that comprises 85 species and 11 subspecies (Williams 1972). Members of the genus have been classified into two sections (*Alatosperma* and *Leucadendron*) by Williams (1972) based on fruit characteristics. *Leucadendron* are successfully cultivated in a number of countries including the United States of America, Australia and New Zealand. In Australia, these plants are popular garden plants and are grown commercially for both the domestic and export cut-flower industries. The availability of molecular markers for assessing variation and relatedness of *Leucadendron* species would greatly aid the development of new hybrids.

A thorough understanding on the phylogenetic relationships within *Leucadendron* requires information from all three genomes. *Leucadendron* phylogeny has been inferred from sequence analysis of the internal transcribed spacer (ITS) regions of the nuclear rRNA genes (Barker et al. 2004). Chloroplast DNA (cpDNA) variation has also been used to evaluate interspecific relationships in *Leucadendron* (Pharmawati et al. 2004). Both ITS- and cpDNA- derived phylogenies disagree substantially with the previous morphological classification of *Leucadendron* species (Williams 1972). The phylogenies based on molecular evidence also differ from one another. To further define the phylogeny of *Leucadendron*, information from mtDNA would be beneficial.

The use of cytoplasmic DNA sequences as breeding, phylogenetic or phylogeographic markers requires knowledge of the mode of inheritance and the level of sequence variation. In the genus *Leucadendron*, the chloroplast genome is maternally inherited and contains useful amounts of sequence variation (Pharmawati et al. 2004). In contrast, the mode of inheritance of mtDNA in *Leucadendron*, as well as the phylogenetic information contained within it, has not yet been examined.

In this study, universal primers specific to land plant mitochondrial genomes (Demesure 1995; Dumolin-Lapegue et al. 1997) were tested for their ability to amplify specific mtDNA fragments from seven *Leucadendron* species. The resulting fragments were subjected to RFLP analysis to evaluate the applicability of this method to the detection of mtDNA sequence variation in *Leucadendron*.

MATERIALS AND METHODS

Plant materials

Leaf tissue from *Leucadendron discolor* Buek ex Meisn, *L. eucalyptifolium* Buex ex Meisn, *L. gandogeri* Schinz ex Gandoger, *L. laureolum* (Lam.) Fourcade, *L. procerum* (Salisb. ex Knight) Williams, *L. salignum* Berg and *L. uliginosum* R.Br were collected from the living collection of the *Leucadendron* Breeding Program, University of Western Australia (Perth, Australia). Voucher specimens were lodged to Australian National Herbarium, Centre for

Australian National Biodiversity Research with voucher number CANB668317, CANB668319, CANB668324, CANB668332, CANB668337, CANB668342 respectively to samples stated above.

DNA extraction and electrophoresis

DNA was extracted from 0.1 g of leaf tissue using a commercial kit (DNeasy plant mini kit, Qiagen, Clifton Hill, VIC, Australia) according to manufacturer's instructions. Genomic DNA and DNA fragments were size fractionated by electrophoresis on agarose gels and visualized by ethidium bromide staining (Sambrook et al. 1989). To determine DNA concentration, the ethidium bromide staining intensity of specific bands was compared to that of a range of co-fractionated lambda DNA mass standards (MBI, Fermentas, Hanover, MD, USA).

PCR-RFLP analysis

Each 25 µL polymerase chain reaction (PCR) assay contained 20 ng DNA template, 16 mM $(NH_4)_2SO_4$, 67 mM Tris-HCl, pH 8.8, 0.01% (v/v) Tween-20, 1.5 mM $MgCl_2$, 0.3µM each primer (Table 1, synthesized by Invitrogen Life Technologies, Mount Waverley, VIC, Australia), 200 µM each standard dNTP and 1 unit of *Taq* DNA polymerase (Bioline, Alexandria, NSW, Australia). The program for the thermal cycler (iCycler, Bio-Rad, Regents Park, NSW, Australia) had an initial denaturation step of 4 min at 94 ^oC, followed by 30 cycles of 45 sec at 94 ^oC, 45 sec at 47^oC to 59 ^oC (depending on the primers used, Table 1) and 2 to 4 min (depending on the product length, Table 1) at 72 ^oC, with a final extension step of 10 min at 72 ^oC.

Aliquots of each amplification product were digested singly with *Alu*I, *Cfo*I, *Hae*III, *Mae*I, *Nde*II, *Rsa*I, *Taq*I, *Tha*I (Promega, Annandale, NSW, Australia), *Hindf*I, *Msp*I or *Mva*I (Roche Diagnostic, Castle Hill, NSW, Australia), but not every fragment was digested with every enzyme. Digestion was for 3 h at 37 ^oC in 10 µl 1 x buffer (supplied by enzyme manufacturer) containing 2 units of enzyme.

DNA sequencing and analysis

The PCR-amplified *nad*1/B-C intron was sequenced directly with dideoxynucleotide chain termination chemistry (BigDye v3.1, ABI, Western Australia) using *nad*1 exon B-C primer pair (Table 1). The products were separated and analysed by The West Australian Genome Resource Centre. The sequences were deposited in GenBank (Accession numbers *L. discolor*, DQ250042; *L. eucalyptifolium*, DQ250043; *L. gandogeri*, DQ250044; *L. laureolum*, DQ250045; *L. procerum*, DQ250046; *L. salignum*, DE250047; *L. uliginosum*, DQ250048). The sequencing data was assembled using AssemblyLIGN (Accelrys, Sydney, Australia) and aligned using ClustalW (MacVector 8.0, Accelrys) software. Multiple sequence alignments generated with ClustalW were used to generate phylogenetic trees using DNApars-PHYLIP (Felsenstein 1989).

Sequence diversity was calculated using MEGA4 (Tamura et al. 2007). For comparison, diversity of *nad1*/B-C sequences from *Actinidia* species was calculated by extracting sequences from GenBank (AJ536471.1; AJ536472.1; AJ536470.1; AJ536469.1; AJ536467.1; AJ536468.1).

RESULTS AND DISCUSSION

Seven 'universal' PCR primer pairs designed to amplify mtDNA fragments from land plants (Demesure et al. 1995; Domulin-Lapegue et al. 1997) were used in this study. The mtDNA regions evaluated were six introns from the genes encoding subunits 1, 4, 5 and 7 of NADH dehydrogenase and the intergenic region between the *rps14* and *cob* genes (Table 1). After optimizing the reaction conditions, each primer pair robustly amplified a single fragment (Table 1) of 1,500 to 4,500 bp, depending on the primer pair, from *Leucadendron* genomic DNA. Amplification products from seven *Leucadendron* species using the *nad*7/2-3r mtDNA primer pair are shown in Figure 1.

The fragments amplified from *Leucadendron* were similar in size to those amplified from other plants using these primer pairs (Demesure et al. 1995; Dumolin-Lapegue et al. 1997; Vaillancourt et al. 2004). The seven amplified fragments totaled about 17,500 bp of *Leucadendron* mtDNA, but no length polymorphisms were detected among the seven species.

Intergenic and intronic regions were chosen for analysis because these regions of mtDNA are less constrained than their adjacent exons for both overall number of substitutions per site and indels (Laroche et al. 1997), and therefore demonstrate higher rates of polymorphism than the very well conserved exonic regions (Duminil et al. 2002). It was therefore surprising that polymorphisms were not detected.

Table 1. Primers and primer pairs used in this study

Primer pair	Annealing Temperature (^oC) [a]	Extension Time (min)[a]	Approximate product size in this study (bp)	References
*nad*1 exon B *nad*1 exon C	57.5	2	1700	Demesure et al. 1995
*nad*4 exon 1 *nad*4 exon 2	59	2	2000	Demesure et al. 1995
*rps*14 *cob*	59	2	1500	Demesure et al. 1995
*nad*5/1 *nad*5/2r	57.5	3	2600	Dumolin-Lapegue et al. 1997
*nad*1/4 *nad*1/5r	47	3	3500	Dumolin-Lapegue et al. 1997
*nad*7/2 *nad*7/3r	57	2	1700	Dumolin-Lapegue et al. 1997
*nad*4/2c *nad*4/3r	53.5	4	4500	Dumolin-Lapegue et al. 1997

Note: [a]Determined empirically

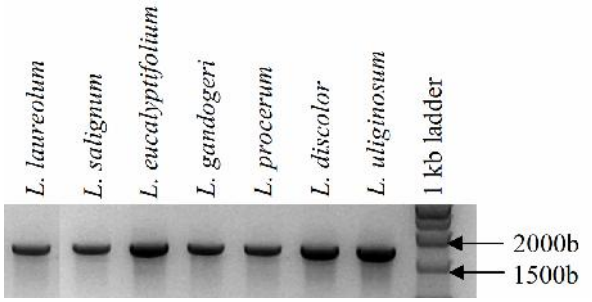

Figure 1. Amplification of the PCR products from seven *Leucadendron* species using the nad7/2-3r mtDNA primer pair. The PCR products were separated on a 1.2% agarose gel electrophoresis and stained with ethidium bromide. The lane containing a 1 kb ladder (Promega) is indicated, as are the sizes of selected marker fragments.

In an attempt to reveal mtDNA sequence polymorphisms across species, each PCR product was digested with a battery of restriction endonucleases having four-base recognition sequences. Sixty-two primer pair - enzyme combinations were tested, revealing a total of 248 discernable restriction sites for each species, directly assaying variation at 992 positions within the mtDNA sequence of each species. Despite this level of sampling, no sequence variations or length polymorphisms were detected. The monomorphic profiles of the mtDNA fragments obtained with the *nad*1 exon B-C - *Mva*I and *nad*7/2-3r - *Hae*III primer pair - enzyme combinations are representative (Figure 2).

The extreme conservation of *Leucadendron* mtDNA sequence is in sharp contrast to the cpDNA in this genus. In the same species examined here, polymorphisms were detected in cpDNA with 14 of 33 primer pair - enzyme combinations when a sequence space of 16,800 bp was sampled (Pharmawati et al. 2004). At least 120 restriction sites representing 480 bp of cpDNA sequence were evaluated across the 16,800 bp region, resulting in the identification of seven restriction site polymorphisms and 33 indels. Across the seven species, this is a site polymorphism rate of 1 per 480 bp assayed.

In contrast, at least 248 restriction sites, representing 992 bp of sequence across a sequence space of approximately 17,500 bp, were examined in *Leucadendron* mtDNA by PCR-RFLP. The lack of polymorphism suggests that mtDNA sequences are highly conserved among *Leucadendron* species, a conclusion that is supported by no polymorphisms within the 1,434 bp *nad*1/B-C sequence that was determined directly for all seven species of this study. This contrasts strongly to the situation found in other plants. For example, PCR-RFLP analysis of mtDNA intronic regions has been used successfully to detect interspecific polymorphisms in *Actinidia* (Testolin and Cipriani 1997), *Elymus* (Sun 2002), *Musa* (Nwakanma et al. 2003), *Vasconcellea* (Van Droogenbroeck et al. 2004) and *Houttuynia* (Wei et al. 2005), and even intraspecific polymorphisms in *Quercus robur* (Dumolin-Lapegue et al. 1995), *Actinidia deliciosa* (Testolin and Cipriani 1997), *Picea abies* (Grivet et al. 1999), *Solanum tuberosum* (Bastia et al. 2001), *Eucalyptus globulus* (Vaillancourt et al. 2004) and *Buchloe dactyloides* (Gulsen et al. 2005). In a study of eight genotypes of *Eucalyptus globulus*, a mtDNA polymorphism was detected within 7960 bp of sequence space after screening only 36 primer pair-enzyme combinations (Vaillancourt et al. 2004). The number of polymorphisms is expected to be higher when mtDNA evaluation is done across species. For

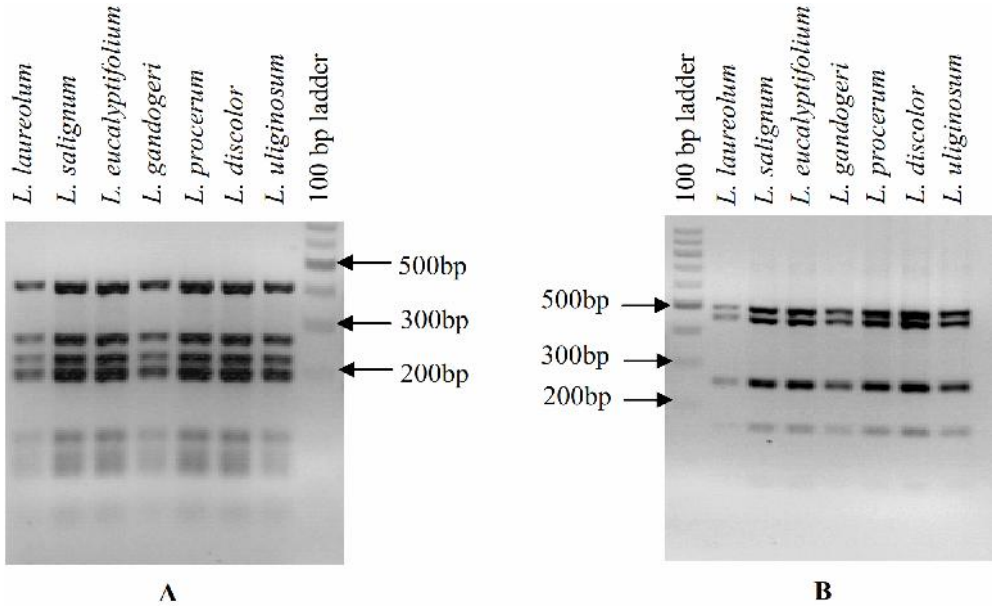

Figure 2. PCR-RFLP patterns of mtDNA from seven *Leucadendron* species. Patterns were generated using the primer pair - enzyme combinations of *nad*1/B-C and *Mva*I (A), and *nad*7/2-3r and *Hae*III (B). The digestion products were separated on a 3% agarose gel electrophoresis and stained with ethidium bromide. Sizes of selected fragments of the 100 bp ladder (Promega) are indicated.

example, among 13 *Elymus* species analysed, polymorphisms were detected within 1600 bp of mtDNA sequence space with two of seven primer-enzyme combinations tested (Sun 2002). The complete inability to detect site variation or indels among seven *Leucadendron* species with 62 primer pair-enzyme combinations indicates the extreme conservation of mtDNA sequence in this genus. Since mtDNA polymorphisms were not found, the inheritance of mtDNA could not be determined by examining the progeny produced by interspecific crosses.

To further investigate the level of sequence conservation in *Leucadendron* mtDNA, the DNA sequence of the 1.5 kb fragment amplified using the *nad*1 exon B-C primer pair was determined for each of the seven species. All seven sequences were identical, demonstrating that the mtDNA sequence is very highly conserved across *Leucadendron* species. The *Leucadendron* sequence was 95% identical to that of the intron located between exons 2 and 3 of the mitochondrial NADH dehydrogenase subunit 1 gene of *Lamprocapnos spectabilis* (AY674682), confirming the sequence identity of *nad*1/B-C. Comparison of the *Leucadendron* and *Lamprocapnos spectabilis* sequences revealed that the length of the intron in the seven *Leucadendron* species tested was 1,434 bp. Each *Leucadendron nad*1/B-C intron had characteristics typical of plant group II introns. There was a GCGCG motif near the 5' splicing site and a domain V sequence (GAGCCACATGCAGGGAAACTTGCACGTGTGGTT) near the 3' splicing site (Chapdelaine and Bonen 1991).

Direct sequencing of the *nad*1/B-C intron showed complete conservation among the seven *Leucadendron* species examined. The nucleotide diversity (π) among samples was 0 and the over all main distance between samples was also 0. In contrast, the mtDNA *nad1*B/C sequences from *Actinidia* species extracted from GeneBank showed nucleotide diversity of 0.312 and the over all main distance between samples was 0.313. The mtDNA *nad1*/B-C intron showed sufficient sequence variation to distinguish among the *Cucurbita* species and allow the construction of a phylogenetic tree (Sanjur et al. 2002). Moreover, intraspecific sequence variation was detected between *C. pepo* subspecies, as well as within *C. moschata* and *C. sororia* (Sanjur et al. 2002).

Leucadendron is a genus which is much younger than other genera in Proteaceae. Using molecular dating, it was reported that *Leucadendron* evolved 40 millions year ago. (Barker et al. 2007). *Actinidia* is dated even younger, approximately 20-26 million year ago (Qian and Yu 1991). However, polymorphism in mtDNA of *Actinidia* was higher. Several repeated motifs within *nad1*B/C were detected (Chat et al. 2004). This was predicted due to hybridization or introgression event as well as high polyploidization in *Actinidia* (Chat et al. 2004). Extensive studies on mtDNA of *Leucadendron* have not been available. This report is the first report on exploration of mtDNA in *Leucadendron* which shows extreme mitochondrial DNA sequence conservation.

The *Leucadendron nad*1/B-C sequence was compared to that of other plants (Figure 5.3). The most parsimonious phylogenetic tree shows that *Leucadendron* (Proteaceae) is most closely related to *Lamprocapnos spectabilis* (Fumariaceae) and *Liriodendron tulipifera* (Magnoliaceae).

The most parsimonious phylogenetic tree obtained using the *nad*1/B-C intron sequence showed that *Leucadendron* is closely allied to *Lamprocapnos spectabilis* (Fumariaceae) and *Liriodendron tulipifera* (Magnoliaceae) and is part of a clade that contains the Platanaceae (*Platanus occidentalis*). Phylogenetic analyses based on *rbc*L cpDNA sequence data (Chase et al. 1993) and combined *rbc*L and *atp*B cpDNA sequences (Drinnan et al. 1994) revealed that Proteaceae was sister to Platanaceae. The result from this study demonstrated that *Leucadendron* and *Platanus* are not sister to one another. However, *Leucadendron* and *Platanus* are part of the same clade and consensus tree of bootstrapped trees showed that the grouping of *Leucadendron* to a clade consisting of *Laurus*, *Platanus* and *Liriodendron* was 979.4 times out of 999.99 tress. Thus, this preliminary analysis of phylogeny based on mtDNA sequence data supports the suggestion that the Proteaceae is placed in a clade with Platanaceae as suggested by Chase et al. (1993) and Drinnan et al. (1994). This placement of the Proteaceae differs from the classification by Johnson and Briggs (1975) based on morphological, anatomical and developmental studies which suggested that the Proteaceae were not closely related to any of the major dicotyledonous orders. Further studies using other markers are required to resolve the position of Proteaceae within the angiosperm lineage.

CONCLUSION

Evaluation of organellar DNA variation showed that no variation could be detected in mtDNA of seven *Leucadendron* species tested using PCR-RFLP and sequence analysis. This indicates that mtDNA is extremely well conserved. Comparison with nad1/B-C sequences from other plants with high homology to that of *Leucadendron* resulted in a phylogenetic tree which shows that *Leucadendron* is clade to *Platanus*.

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