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Simple PCR Markers for the Study of Chloroplast in *Eucalyptus*

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Summary

Combined use of two newly designed PCR primers with already described *rpl*2 and *trn*H primers, yields amplification of three non-independent products from the hypervariable J_{LA} region of eucalypt chloroplast. Polymorphism analysis of the resulting PCR markers is proved to be a time- and cost-efficient alternative to traditional cpDNA techniques as RFLP or sequencing for *Eucalyptus globulus* Labill. population genetics studies.

Key words: Eucalyptus; chloroplast DNA; J_{LA} junction; PCR markers.

Introduction

First studies on chloroplast DNA (cpDNA) of *Eucalyptus* used RFLP as molecular markers (JACKSON *et al.,* 1999; STEANE *et al.,* 1998). For large sample sizes, the effort and time needed to obtain the required quality and amount of DNA may become a serious drawback of the technique. Later on, VAILLANCOURT and JACKSON (2000) discovered the hypervariability of the region surrounding the J_{LA} junction between the large single copy region (LSC) and one of the inverted repeats (IR) of eucalypt DNA and pointed out to its usefulness to approach studies in the genus. Sequence analysis of this region has been used in posterior works revealing the complex evolution pattern of eucalypts (FREEMAN *et al.,* 2001; MCKINNON *et al.,* 2001). The present work describes a simplified method to analyse this hypervariable region (HVR), by following the differential display of three non-independent PCR-products. Our results suggest the usefulness of the method to approach phylogeographic and population studies, avoiding expensive and time-consuming techniques as RFLP or sequencing.

Material and Methods

Thirty plants from three of the original races defined by DUTKOWSKI and POTTS (1999) (South-Eastern Tasmania, Furneaux Group and Western Otways, collecting ten samples in at last two different populations from each race) of *E. globulus* ssp. *globulus* were analysed. Total DNA was extracted from leaves following the method described by DOYLE and DOYLE (1990).

Based on the sequence published by VAILLANCOURT and JACKSON (2000), two primers were designed: HVR1, 5'TAGGAGTAATTAATGG, and HVR2, 5'CCATTAATTACTCC, (corresponding to the positions 132-147 and 147-134, respectively, in the mentioned sequence). Two additional primers, developed by GOULDING et al. (1996) were also used in this approach: *rpl*2, 5'GATAATTTGATTCTTCGTCGCC, and trnH, 5'CGGATG-

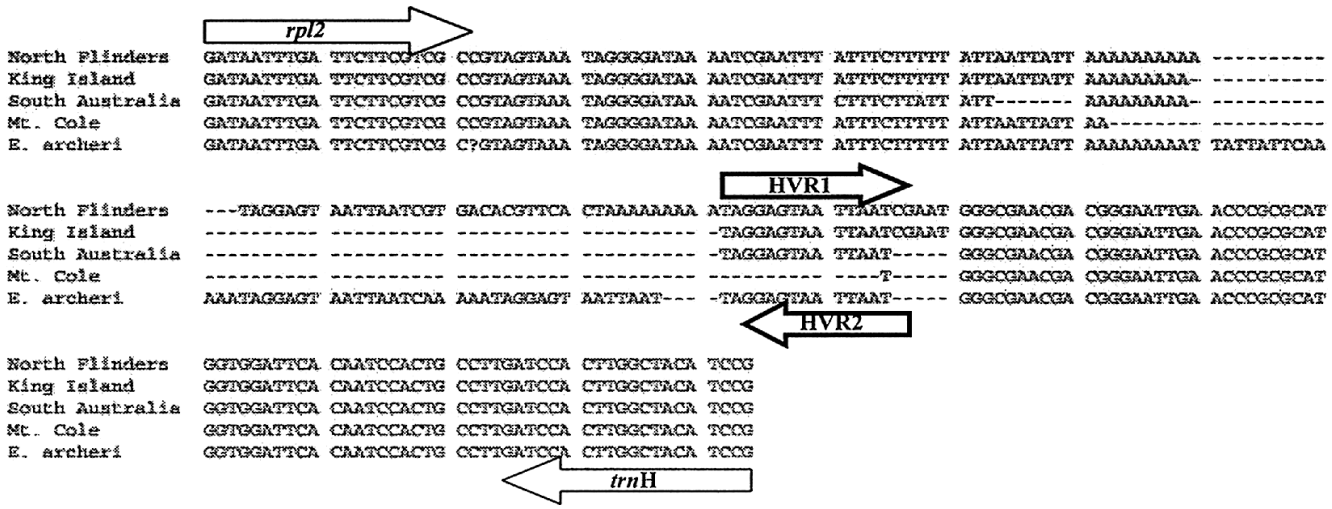

Figure 1. – DNA sequence of the hyper variable J_{LA} region of the chloroplast in three provenances of *Eucalyptus globulus* and one *E. archeri*, according to VAILLANCOURT and JACKSON (2000). Arrows indicate the location of the primers used in the present study.

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Table 1. – Observed and theoretical haplotypes, deduced from the sequence published by VAILLANCOURT and JACKSON (2000). Sizes of the fragments are expressed in base pairs.

Observed haplotype name	Observed			Theoretical		
	*rpl2-trn*H	HVR1-*trn*H	*rpl2*-HVR2	*rpl2-trn*H	HVR1-*trn*H	*rpl2*-HVR2
A	210	130/93/88	132/95	212	132/94	134/96
B	171	93/88	95	173	94	95
C	171	93	95	168	89	95
D	165	88	94	167	89	94
				162	89	89
				161	89	88
				148	-	-

Table 2. – Absolute haplotype frequencies detected in the populations included in the study.

Haplotype / Provenance	A	B	C	D
Western Otways	0	0	10	0
South-Eastern Tasm.	0	0	4	6
Furneaux Group	2	8	0	0

TAGCCAAGTGGATC (*Figure 1*).

Primer combinations *rpl*2-*trn*H, HVR1-*trn*H, and *rpl*2-HVR2 were used to amplify three non-independent fragments. PCR reactions were performed in a final volume of 10 µl, with 5 ng of total DNA, 0.15 µM of each primer, 0.2 mM dNTPs, 50 mM KCl, 10 mM Tris/HCl pH 9, 2 mM $MgCl_2$ and 0.4 U of Taq-DNA polymerase (Ecogen). Standard amplification profile consisted on a first denaturalisation step at 94°C for 5', followed by 20 amplification cycles of 94°C 1', 51°C 30", 72°C 1', and a final elongation step at 72°C for 10'. HRV2 and *trn*H primers were labelled on the 5' end with IRD 800 (MWG Biotech). Sizes of the amplified fragments were scored on an automatic sequencer Li-Cor 4200, using commercial size standards 50-350 bp (Li-Cor).

Results and Discussion

According to the sequences published by VAILLANCOURT and JACKSON (2000), corresponding to *Eucalyptus globulus* from twenty-one different Australian locations, the amplification of the fragments *rpl*2-*trn*H, HVR1-*trn*H and *rpl*2-HVR2 would allow the detection of up to seven different patterns or haplotypes. The analysis of 30 trees representing 3 races of *E. globulus* spp. *globulus* (DUTKOWSKI and POTTS, 1999) yielded a total of four haplotypes (*Table 1*). A discrepancy of 1-2 bp was observed between the sizes of the amplified fragments and the theoretical ones, probably due to the scoring system. More striking is the simultaneous presence of the 93 and 88 bp products for the HVR1-*trn*H fragment in the observed haplotypes A and B. This pattern was only detected in all the samples from Furneaux Group (Central Flinders Island), and therefore a scoring mistake or a contamination can be denied. The most likely explanation for this variant is the presence of a secondary target for the HVR1 primer, due to a duplication event, similar to those ones described by VAILLANCOURT and JACKSON (2000). A feasible mechanism for such duplications in the junctions JLA and JLB of the chloroplast of angiosperms is provided by GOULDING *et al.* (1996). VAILLANCOURT and JACKSON (2000) report the presence of a duplication of the HVR1 and 2 targets in large insertion (positions 94 to 131) in the North Flinders sample included in their study. Such insertion brings out the theoretical variant of 212 bp for the *rpl*2-*trn*H fragment and the associated 134/96 bp and 132/94 bp variants for the *rpl*2-HVR2 and HVR1-*trn*H fragments, respectively. In our analysis 2 out of the 10 samples of the Furneaux Group, region which North Flinders belongs to, showed the corresponding pattern (A haplotype), while the other eight show the B haplotype.

Table 2 shows the distribution of haplotypes detected in the samples studied. Otway Ranges samples showed the expected pattern, according to VAILLANCOURT and JACKSON (2000); six of the South-Eastern samples, from Ellendale, showed the pattern expected for the Triabunna location (also belonging to South-Eastern Tasmania race), according to the above mentioned work, while the other four, from Moogara, showed the same haplotype as Otway Range samples.

Conclusion

The present work reports a novel method for the analysis of the hypervariable region surrounding the J_{LA} junction of *E. globulus* chloroplast. The combined analysis of three simple PCR markers allows an easy and cost-efficient characterisation of this region, avoiding RFLP or sequencing. Consistency of the results presented here shows the suitability of the method for inter-provenance studies in *E. globulus,* and points out to its usefulness for analyses at more detailed levels, as suggested by the variability detected within South-Eastern and Furneaux races.

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