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Identification of *Pinus elliottii* var. *elliottii* X *P. caribaea* var. *hondurensis* Hybrids Using the Chloroplast *trnL-F* intergenic Spacer

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Summary

Breeding programs based on hybrids of tree species often have a need to unambiguously distinguish intraspecific and self progeny from hybrid progeny. The interspecific hybrid of *P. elliottii* var. *elliottii* (PEE) and *P. caribaea* var. *hondurensis* (PCH) is difficult to reliably distinguish from pure PEE based on morphology, especially at a young age. We examined the *trnL-F* intergenic spacer region of the paternally inherited chloroplast genome for a polymorphism that may distinguish these two taxa. Sequencing this region indicated there were two haplotypes, one which was specific to PEE, designated (+), the other designated (–), was shared by both taxa. This result was consistent with other studies which suggest past introgression of PCH into PEE. A PCR assay was developed to detect the PEE specific haplotype. This haplotype was found at a frequency of 0.6 in a sample of 22 PEE from a breeding population but was absent from a sample of 30 PCH parents. As expected, hybrids from crosses of PCH pollen donors and maternal (+) haplotype PEE had (–) haplotypes. In situations where the mother can be genotyped, and the pollen pool consists of maternal (self) or PCH pollen, this assay would unambiguously determine the hybrid status of offspring from 60 % of the PEE

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parents tested. Where paternal candidates include other PEE, the assay would conclusively identify non-hybrid progeny at a lower percentage determined by the frequency of the (+) haplotype in the pollen pool. The chloroplast *trnL-F* spacer assay would be useful for verifying hybrids from future seed orchards based on mother trees with (+) haplotype or for (+) haplotype mother trees in current orchards. The assay will also confirm suspected pure PEE individuals where they have a (+) haplotype but will be inconclusive where they have a (–) haplotype.

Key words: conifers, gymnosperms, PCR, assay, interspecific hybridisation.

Introduction

Plantation forestry in Queensland is predominantly based on *Pinus elliottii* var. *elliottii* (PEE) and *P. caribaea* var. *hondurensis* (PCH) and their interspecific hybrid (PEH) (HAINES, 2000). The hybrid is used to replant areas previously planted to PEE and PCH because of its superior attributes for structural timbers (NIKLES, 1996; TOON *et al.,* 1996). The hybrid is typically produced by pollinating a PEE mother with PCH pollen and deployed as select clones, or in the past as controlled-pollinated F_1 or backcross families. However, incomplete control of pollination during operational seed production may lead to reduced productivity if this inadvertently results in planting PEE. The productivity of pure PEE may be further reduced due to the effects of inbreeding depression in offspring that are from a self pollination or because outcrossed offspring of contaminant PEE pollen-donor parents are sub-optimal for hybrid sites.

A reliable method to identify interspecific hybrids from intraspecific offspring would be valuable both for quality control during the production of hybrid planting stock and in the optimisation of seed orchard design. Identification of hybrids based on morphology is difficult, particularly at an early age and whilst some roguing of pure PEE offspring has been performed in the nursery, this is not completely effective as both intraspecific and self offspring have been detected in field trials and plantations (unpublished data).

DNA markers are increasingly important for hybrid detection in synthetic and natural populations (RIESEBERG, 1998). Tests based on DNA markers tend to be more reliable than morphological traits, as they are not influenced by environment, are generally non-destructive, reproducible and require only small amounts of tissue. Tests based on variants in uniparental, haploid organelle markers, such as the chloroplast, may be more simple and definitive than diploid nuclear markers in outcrossing tree species where the average individual is typically highly heterozygous. The chloroplast genome is paternally inherited in conifers, hence, is effectively a pollen marker (CATO and RICHARDSON, 1996; NEALE and SEDEROFF, 1989). The chloroplast has been well studied in conifers and a complete genome sequence and mono-nucleotide microsatellite markers are available (CATO and RICHARDSON, 1996; VENDRAMIN *et al.,* 1996; WAKASUGI *et al.,* 1994). The chloroplast has a comparatively low evolutionary rate compared with the nuclear genome in plants and has been widely used for investigation of interspecific relationships (GIELLY and TABERLET, 1994; TABERLET *et al.,* 1991). Previous study of chloroplast variation of PEE and PCH has supported the close affinity of these taxa and probable past introgression of PCH into PEE as they shared the second most common PEE haplotype (NELSON *et al.,* 1994).

In this study, we examine variation in the *trnL-F* intergenic spacer of the chloroplast as a candidate locus for identifying interspecific hybrids of PEE and PCH. This locus has previously been reported to be polymorphic between species of *Pinus* (GIELLY and TABERLET, 1994). We report on sequence variation at this site and frequencies of haplotypes in each taxon, and a polymerase chain reaction (PCR) assay which was used to detect interspecific hybrids and self progeny of PEE and then discuss its possible applications.

Methods

Plant Material

Our objective for sampling was to include a broad representation of the Queensland Forest Research Institute (QFRI) PEE and PCH breeding populations. Foliage was collected from trees in trials, plantations and seed orchards for 22 PEE and 30 PCH parents. Samples of both breeding populations were biased toward individuals that were prominent parents for the production of F_1 hybrids. It was possible to structure sampling of the PCH population to include material from coastal, island and inland provenances from its native range, as the provenance of PCH material introduced into Queensland was usually known. However, this was not possible with PEE, as almost all PEE introduced into Queensland was in the form of mixtures of many seed parents from stands in north Florida or Georgia in the USA. Seven interspecific hybrids (PEH) and several putative "selfs" of a PEE were also sampled. A list of the parents and hybrid offspring is provided in *Table 1.*

Table 1. – List of *Pinus elliottii* var *elliottii, P. caribaea* var *hondurensis* and hybrid samples with their *trnL-F* spacer haplotypes as determined by sequencing and a PCR assay.

Genotype identifier[1]	Sequence polymorphism[2]	Haplotype by PCR[3]	Genotype identifier[1]	Sequence polymorphism[2]	Haplotype by PCR[3]
1ch1-063	12T/TACC	-	1ee1-086	na	-
1ch1-113	na	-	1ee1-161	na	-
1ch4-057	12T/TACC	-	1ee2-037	na	-
1ch4-078	na	-	1ee2-043	na	-
1ch4-089	na	-	1ee2-057	12T/TACC	-
1ch4-148	na	-	1ee2-015	na	-
1ch4-221	na	-	1ee3-015	12T/TACC	-
1ch4-240	na	-	1ee1-015	na	+
1ch4-243	na	-	1ee1-074	na	+
1ch4-245	na	-	1ee1-142	na	+
1ch4-247	na	-	1ee2-006	na	+
1ch4-249	na	-	1ee2-012	na	+
1ch4-251	na	-	1ee2-031	11T/GGTA	+
1ch4-254	na	-	1ee2-048	na	+
1ch4-257	na	-	1ee2-081	na	+
1ch4-258	na	-	1ee3-005	na	+
1ch4-326	na	-	1ee3-027	na	+
1ch4-327	na	-	2ee1-088	na	+
1ch6-002	12T/TACC	-	2ee1-102	11T/GGTA	+
1ch6-029	12T/TACC	-	2ee1-166	11T/GGTA	+
1ch6-059	12T/TACC	-	2ee1-105	12T/TACC	-
1ch6-089	12T/TACC	-	2ee1-107	12T/TACC	-
1ch6-148	na	-	eh43	na	-
1ch6-222	na	-	eh49	na	-
2ch4-129	na	-	eh83	na	-
2ch4-151	na	-	eh88	na	-
2ch4-157	na	-	eh89	na	-
2ch4-167	na	-	eh90	na	-
2ch4-198	na	-	p18 r4t11	na	-
2ch4-203	na	-			

[1]) Genotype identifier:- 1st digit refers to selection generation, ee = *Pinus elliottii* var *elliottii, ch = Pinus caribaea* var *hondurensis;* 2nd digit is the region designation in Queensland from where selection was made; Number following dash is selection number. An "eh" in the identifier indicates a select PEE X PCH hybrid. Sample "p18 r4t11" was an individual putative hybrid from a clone bank, Byfield Queensland.

[2]) Haplotype determined by sequencing. Two haplotypes were identified. One haplotype had a 11 bp mono-thymidine repeat beginning at position 113 that was linked to a GGTA substitution at position 344-347. The second had a 12 bp thymidine repeat that was linked to a TACC substitution at the same position.

[3]) A "+" indicates a PCR product of 364 bp was generated in a PCR assay with a primer specific to the "GGTA" haplotype. A "–" indicates the PCR product was not amplified from these individuals.

DNA extraction

Fresh needles were collected and stored chilled prior to and during transport. Needles were then stored at –20 °C prior to DNA extraction. Purified DNA was prepared from 50 mg of tissue using the DNeasy 96 plant extraction method (Qiagen), as described in SHEPHERD *et al.,* 2002 or using a CTAB method (BOUSQUET *et al.,* 1990). DNA was quantified by comparison with standards on agarose gel and diluted to 20 ng/ul for PCR.

DNA sequencing

Direct sequencing of the intergenic spacer between the tRNA genes, *trnF* and *trnL* from the large single copy region of the chloroplast was performed as follows; the intergenic spacer region was amplified with primer (E) 5'-GGTTCAAGTCCCTC TATCCC-3' (TABERLET *et al.,* 1991) and primer (F) 5'ATTT GAACTGGTGACACGAG-3' using the conditions described in GIELLY and TABERLET (1994). This product was purified by ethanol precipitation and quantified by comparison with standards on a 1.5 % agarose gel stained with ethidium bromide. Forward and reverse sequencing templates were prepared according to the Perkin Elmer ABI Prism™ BigDye™ Terminator Cycle Sequencing Manual (Perkin Elmer, Foster City, CA). Gel separation was performed using an ABI 377 Automated DNA sequencer (ABI PE Foster City, CA) by the Australian Genome Research Facility, Brisbane Australia or by Sydney University and Prince Alfred Macromolecule Analysis Centre, (Sydney, Australia). Sequence alignment was carried out using MacVector v6 software (Oxford Molecular Group, Oxford).

Chloroplast trnL-trnF intergenic spacer assay

A PCR assay which specifically detected the PEE haplotype was developed by designing a primer (5'-GTCAACTAAAAA GAAGTAAAAATACC-3') whose 3' end terminated on the 4 base-pair (bp) polymorphic region (344-347 bp) and that was thermodynamically compatible with primer (E). 25 µl PCR reactions consisted of 20 ng of template, 1x Boehringer Mannheim PCR buffer, 0.5 U Taq, 0.8 mM total of 4 dNTP's, primers and additional 1 mM MgCl2. The reaction included 2 reverse primers, primer F (0.33 mM) and "PEE cp *trnL* spacer A (1 mM) and one forward primer, primer E (1 mM). A "touchdown" cycling program was carried out on a PE 9600 thermocycler. The program consisted of an initial denaturation at 93 °C for 45 s, followed by 20 cycles of 93 °C, 60-50 °C decreasing by 0.5 °C per cycle, 72 °C each for 30 s followed by 20 cycles of 93 °C for 30 s, 50 °C for 30 s and 72 °C for 1 min. Products were separated on 1.5 % agarose and visualised by staining with ethidium bromide.

Results

Sequence of the trnL-F spacer in PEE and PCH

Variation in a 470 base-pair fragment of the intergenic-spacer region between the *trnL* and *trnF* genes on the chloroplast was studied by sequencing eight PEE and six PCH samples (*Table 1*). Two haplotypes were identified, one specific to PEE and the other shared by PEE and PCH parents (GenBank Accession numbers AF 528523 and AF 528522 (in this order) respectively). The PEE specific haplotype was found in four of the eight PEE. The remaining four PEE possessed a second haplotype which was found in all six PCH. The haplotypes differed at two sites, the first polymorphism occurred at base-pair position 113 and consisted of a change in the length of a thymidine mono-nucleotide element from 12 bp to 11 bp in the PEE specific haplotype (*Figure 1*). The second polymorphism occurred at the base-pair positions 344–347 bp and consisted of a 4 bp substitution. These polymorphisms represent a 0.4 % sequence divergence between haplotypes. The two haplotypes were aligned with the *trnL-trn*F spacer from two other *Pinus*, *P. sylvestris* (Genbank Accession X76821 (181bp) (GIELLY and TABERLET, 1994)) and *P. uncinata* (Genbank Accession X76825 (102bp) (GIELLY and TABERLET, 1994)) to confirm orthogonality of our sequences. Sequence divergence between the PEE specific haplotype and these species was 6.6 % and 1.0 % respectively.

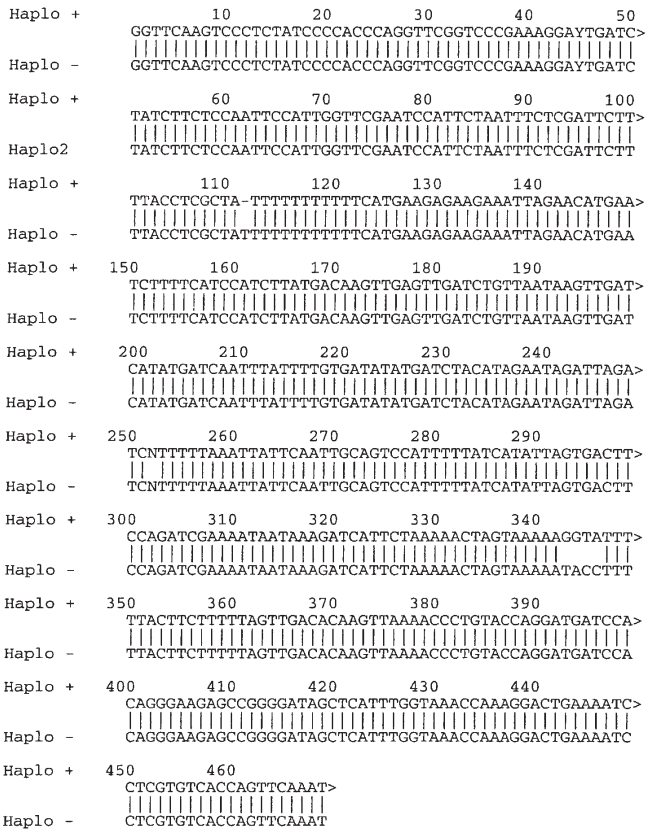

Figure 1. – Sequence of haplotypes identified in PEE and PCH

A PCR assay for a PEE specific haplotype

A PCR primer was designed to specifically complement the PEE specific haplotype in the 344-347 bp region. A sample of 22 PEE, 30 PCH and 7 PEH individuals, which included those previously tested, were assessed for amplification of a 364 bp fragment diagnostic of the PEE specific haplotype (*Table 1* and *Figure 2*). This assay also included a PCR positive control, whereby a product of 470 bp PCR product was generated when conserved priming sites were present in the template and the reaction conditions were suitable for PCR. The control fragment was amplified from all samples. All PCH individuals were designated as a (–) haplotype, as there was no amplification of the 364 bp diagnostic fragment. Thirteen (59 %) of the twenty-two PEE individuals tested, amplified the diagnostic fragment and were designated as (+) haplotypes. This group included the four PEE individuals previously identified by sequencing as having the PEE specific haplotype. The nine remaining PEE individuals failed to amplify the diagnostic fragment, and were designated as (–) haplotypes. The seven hybrids did not amplify the diagnostic fragment, which was consistent with the paternal inheritance of the (–) haplotype from their PCH parent. Two individuals which were putative selfed progeny of a (+) haplotype PEE maternal parent were also tested and

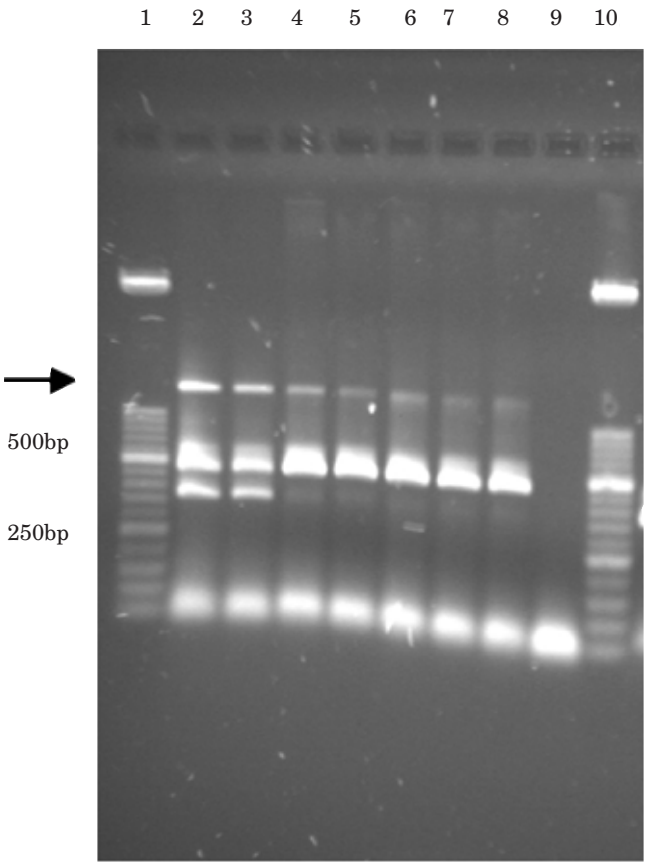

Figure 2. – Discrimination of chloroplast *trnL-trnF* intergenic-spacer haplotypes for PEE, PCH individuals and their hybrids. Lanes 1 & 10 = Molecular weight standard, 50 bp ladder (Boehringer Mannheim), 2 = 1ee1-015, 3 = 1ee2-031, 4 = 1ch6-002, 5 = 2ch6-029, 6 = 2ee1-107, 7 = PEE x PCH hybrid 1, 8 = PEE x PCH hybrid 2, 9 = Negative control reaction ie no. DNA template. The 364 bp fragment was diagnostic of the PEE specific haplotype. A fragment at approximately 470 bp was a "positive" control band that indicated the conditions were suitable for PCR. Arrow indicates a non-specific amplification product at position > 1 kb.

found to have (+) haplotypes (data not shown). These individuals had previously been fingerprinted with random amplified polymorphic (RAPD) markers which suggested they were selfed progeny (unpublished).

Discussion

We examined the *trnL-F* intergenic spacer region of the chloroplast genome as a possible marker to distinguish the interspecific hybrids of PEE X PCH from progeny arising from a self fertilisation of a PEE. Our objective was to develop a technically simple and cost effective assay that would assign PEE progeny into the two pools, hybrids and non-hybrids. We found two haplotypes amongst the PEE and PCH tested, one haplotype which was specific to 59% of PEE parents. An assay was developed that could unambiguously determine self from hybrid progeny for the (+) haplotype PEE parents where the pollen pool consisted of maternal (self) pollen or PCH pollen and the maternal tree was available for haplotyping (*Table 2a*). If the pollen pool also potentially contains non-maternal tree PEE pollen, then the assay would only detect non-hybrid progeny with a (+) haplotype with certainty. The frequency of a definitive assignment would depend upon the frequency of the (+) haplotype in the pollen pool. Assuming that parental haplotypes occur at the frequencies detected in this study, and that each pollen donor contributes to the pollen pool equally and that all progeny are viable, 17 % of progeny would, on average, be identifiable as non-hybrids for PEE (+) mothers and 12 % percent for PEE (–) mothers (*Table 2b*).

This chloroplast *trnL-F* spacer assay should be useful in applied breeding and research applications for verifying hybrids from future seed orchards based on mothers with a (+) haplotype or for (+) haplotype mothers in current orchards. The assay will also allow the elimination of pure PEE individuals from pollen donors with (+) haplotypes but will be inconclusive where offspring have a (–) haplotype. The assay is relatively simple and cost effective, requiring only basic molecular biology laboratory equipment. The assay has the advantages of a PCR based assay, requiring only small amounts of tissue that

Table 2a. – Frequency of unambiguous hybrid determinations using the chloroplast PEE haplotype assay when the maternal haplotype is known and candidate pollen donors include the maternal (self) PEE or PCH trees.

Table 2b. – Frequency at which non-hybrid progeny can be detected using the chloroplast PEE haplotype assay when the maternal haplotype is known and candidate pollen donors include the maternal (self) PEE, other PEE or PCH trees.

Cross	Maternal haplotype	Offspring haplotype	Conclusion	Assay unambiguous	Maternal haplotype frequency in PEE popn.
PEE+ X PEE+	+	+	self	Y	0.59
PEE+ X PCH-	+	-	Hybrid	Y	0.59
PEE- X PEE-	-	-	Hybrid or self	N	0.41
PEE- X PCH-	-	-	Hybrid or self	N	0.41

Cross	Mother haplotype	Offspring haplotype	Conclusion	Maternal haplotype freq. in PEE popn.	Paternal haplotype freq. in pollen donor popn.[1]	Freq. of matings
PEE+ X PEE+	+	+	Not a hybrid	0.59	0.30	0.17
PEE+ X PEE-	+	-	Inconclusive	0.59	0.71	0.42
PEE+ X PCH-	+	-	Inconclusive	0.59	0.71	0.42
PEE- X PEE+	-	+	Not a hybrid	0.41	0.30	0.12
PEE- X PEE-	-	-	Inconclusive	0.41	0.71	0.29
PEE- X PCH-	-	-	Inconclusive	0.41	0.71	0.29

[1] Frequency of paternal haplotype in pollen donor population will only equal the frequency of pollen haplotypes when there is equal contribution of pollen from each pollen donor and progeny are equally viable.

need not be fresh. It should be possible to implement this assay in an industry laboratory with minimal equipment outlay and using non-specialist technical staff. The (+)/(–) nature of the assay would make it amenable to a high throughput non-gel based detection system.

Although this assay has a number of potential applications, it is also limited in that it is only definitive for maternal parents of known (+) haplotype. A more broadly applicable assay would not require knowledge of the maternal parent and could be based on the detection of a haplotype which is monomorphic and private to PCH. Several hundred nuclear microsatellite markers have been developed for hard pines (Genus *Pinus* subgenus Pinus) (eg. ECHT and BURNS, 1999; ELSIK *et al.,* 2000; FISHER *et al.,* 1996; SMITH and DEVEY, 1994)). Many of these loci transfer to PEE and PCH but around three percent of those transferred are monomorphic (SHEPHERD *et al.,* 2001a). A small number are monomorphic in each taxon but polymorphic between the two taxa for a small number of individuals. Nuclear microsatellite loci which are fixed for different alleles in each parental taxon may provide an alternative hybrid verification assay and overcome the limitations of the current chloroplast assay.

Investigation of other chloroplast loci such as the mononucleotide microsatellite loci may also provide a more broadly applicable assay. A potential problem common to all tests based on chloroplast markers, however, is the possibility of maternal leakage i.e. inheritance of the chloroplast from the mother, which has been found in some *Pinus* spp. (CATO and RICHARDSON, 1996; NEALE and SEDEROFF, 1989). Maternal leakage was not detected in this study of PEE and PCH, and only occurs at low frequencies (1%) in other pines, hence, this is not thought to be a major limitation to the use of chloroplast loci as pollen markers in conifers. The chloroplast *trnL-F* spacer assay should be a useful assay for hybrid identification in a defined range of applications and is attractive because of its simplicity, robustness and low cost.

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