VERIFICATION OF INTERSPECIFIC PINE HYBRIDS USING PATERNALLY INHERITED CHLOROPLAST MICROSATELLITES

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ABSTRACT

Pollen contamination continues to be a problem for many operational controlled-pollination programs for hybrid trees. In the case of the slash × Caribbean pine hybrid in Queensland, Australia, productivity losses may occur if pure slash is deployed on sites optimal for hybrids. We report a DNA marker assay based on chloroplast microsatellite loci that distinguishes between representatives of slash and Caribbean pine breeding populations. Furthermore, interspecific hybrid F_1s were distinguishable from individuals believed to be derived from self fertilisation or outcrossing to other slash pine. The multi-locus assay was optimised for a single-tube PCR reaction and offered an estimated 75% savings in the turnaround time and costs compared with individual PCR amplification of the six loci. Slash pine displayed more variation at these loci than Caribbean pine despite Caribbean pine having been sampled over a more extensive geographical range than the slash pine.

Keywords: *Pinus elliottii* var. *elliottii*, *P. caribaea* var. *caribaea*, hybrid breeding, DNA fingerprinting.

INTRODUCTION

Breeders working with hybrids frequently have a need to unambiguously determine the hybrid status of offspring. Plantation forestry in Queensland is predominantly based on slash pine, *Pinus elliottii* var. *elliottii* (PEE) and Caribbean pine, *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). 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 distinguish 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 is carried out in the nursery, this is not completely effective as both intraspecific and selfed offspring have been detected in field trials and plantations (unpublished data).

The chloroplast in conifers is inherited from the pollen (CATO & RICHARDSON 1996; NEALE & SEDEROFF 1989) which makes it an attractive marker for monitoring the paternal contribution to offspring. The chloroplast genome has a low evolutionary rate compared with the nuclear genome in plants and has been widely used for investigation of interspecific relationships (GIELLY & TABERLET 1994). The low mutation rate, low effective population size and the potential for periodic selection of the chloroplast, mean that its patterns of diversity may also differ markedly to that of the nucleus (HONG *et al.* 1993).

Here we report on chloroplast microsatellite variation in PEE and PCH. We were able to distinguish all PCH from PEE using a multilocus haplotype (MLH) derived from the joint analysis of single-locus haplotypes (SLH). We also validate the paternal pedigree of F_1 hybrid offspring and distinguish amongst

unintentional "selfed" individuals and unintentional intraspecific crosses. This assay extends the power to discriminate between these taxa beyond that of an assay based on the *trn*L-F intergenic spacer of the chloroplast (SHEPHERD & HENRY 2003). We report that chloroplast microsatellites for pines can be conveniently multiplexed.

METHODS

Populations, foliage collection and DNA extraction

A reference population that broadly represented the Queensland Forest Research Institute (QFRI) breeding populations for PEE and PCH taxa was formed from 23 PEE and 29 PCH individuals. PEE were chosen at random from the PEE breeding population (Table 1). PEE was introduced into Queensland as bulked seed lots mainly from collections in north east Florida, south east Georgia region (NIKLES 1996). The PCH parents were selected to represent the main provenance sources used in Queensland. The major sources of PCH for breeding populations in Queensland are uplands of Belize (Mountain Pine Ridge), coastal lowlands of Belize and Guanaja Island provenance (NIKLES 1996). To this reference population of 52 trees, 12 putative F_1 hybrids derived from controlled crosses were also selected to test chloroplast inheritance patterns. Pedigrees of these F_1 are given in Table 1. Where possible, the relevant parents were included in the reference population. PEE was the seed parent in all these crosses. This set of 12 F_1 included two putative F_1, "BS8" and "50.1.12" which previous analysis with nuclear microsatellites had indicated were not progeny of the intended cross (data not shown).

Pine needles were supplied by QFRI and stored frozen at –20 °C. DNA was extracted using a DNeasy 96 plant extraction kit (Qiagen Valencia, CA) after grinding with a Mixer Mill (MM300 F. Kurt Retch GmbH & Co. KG, Hann, Germany) using the protocol for frozen tissue with the following modifications; 800ml of Buffer AP1/RNAse A/Reagent DX was used instead of the recommended 400 ml, foliage was digested at 65 °C for 20 min in Buffer AP1 before adding 260 ml of Buffer AP2. Two aliquots of ~400 ml were retrieved into duplicate sets of microcollection tubes before adding Buffer AP3/E. The two aliquots were sequentially applied to a single silica column per sample.

Pinus spp. chloroplast microsatellite marker amplification

Twenty chloroplast microsatellite primers were reported by (VENDRAMIN *et al.* 1996) based on all the mononucleotide repeat regions identified in the complete chloroplast genome sequence of *Pinus thunbergii* (WAKASUGI *et al.* 1994). In this study, ten primer pairs that generated the smallest PCR fragments were chosen to increase the likelihood of resolving short length polymorphisms (Pt102584, Pt109567, Pt15169, Pt26081, Pt30204, Pt36480, Pt45002, Pt71936, Pt79951 and Pt87268). Unlabelled and 5' fluorescently labelled primers were synthesised by Proligo (Lismore, Australia).

Single locus amplification and assay detection

PCR conditions were based on VENDRAMIN *et al.* (1996) with the following exceptions. Reactions were carried out in 20 ml volumes using 20 ng of total genomic DNA template. Taq polymerase and PCR buffer was supplied by Roche Applied Science (Mannheim, Germany). The concentration of $MgCl_2$ in the Roche 10 × PCR buffer (15 mM) was supplemented with additional $MgCl_2$ to give a final concentration of 2.0 mM. To initially identify which loci were polymorphic, each reaction also included fluorescently labelled dUTP (R110; RG6 or TAMRA, Applied Biosystems) at a final concentration of 0.4 mM. Markers that were identified as polymorphic were subsequently amplified with a forward primer that was re-synthesed with a 5' dye label (Pt109567F-Fam, Pt15169F-Tet, Pt45002F-Hex, Pt79136F-Tet, Pt79951F-Hex and Pt87268F-Fam). Cycling was carried out using a PE 9600 thermocycler (Perkin Elmer) programmed for 25 cycles of 94 °C, 55 °C and 72 °C each for 1 min preceded by a 95 °C hold for 5 min and followed by a hold at 72 °C for 7 min.

PCR products were resolved using capillary electrophoresis and visualised using a fluorescence detection (ABI310 Genetic Analyser, Applied Biosystems). Samples were prepared for 310 analysis by combining 1 ml of PCR product with 17.5 ml of Deironised formamide (Amresco) and 0.5 ml of GS ROX 500 molecular weight standard (Applied Biosystems) before denaturing at 95 °C for 3 min. Samples were analysed on the AB 310 using module A with a five second injection at 15,000 volts with a twenty four min run time at 60 °C. Haplotypes were scored with the aid of Genotyper software v3.71 NT (Applied Biosystems).

Table 1. Multi-locus cp microsatellite haplotypes for 64 PEE, PCH and PEH individuals.

Provenance /Pedigree	Haplotype	Taxa	Genotype	Pt109567	Pt15169	Pt45002	Pt71963	Pt79951	Pt87268
Coastal Plain	1	PCH	1ch1-113	111	106	160	145	133	162
Melinda	1	PCH	1ch4-221	111	106	160	145	133	162
Kuakil	1	PCH	1ch4-240	111	106	160	145	133	162
Karawala	1	PCH	1ch4-243	111	106	160	145	133	162
Melinda	1	PCH	1ch4-251	111	106	160	145	133	162
Alamicamba	1	PCH	1ch4-257	111	106	160	145	133	162
Brus Lagoon	1	PCH	1ch4-326	111	106	160	145	133	162
Brus Lagoon	1	PCH	1ch4-327	111	106	160	145	133	162
Brus Lagoon	1	PCH	1ch6-089	111	106	160	145	133	162
MPR	1	PCH	1ch6-222	111	106	160	145	133	162
MPR	1	PCH	2ch4-129	111	106	160	145	133	162
MPR	1	PCH	2ch4-157	111	106	160	145	133	162
MPR	1	PCH	2ch4-167	111	106	160	145	133	162
MPR	1	PCH	2ch4-198	111	106	160	145	133	162
MPR	1	PCH	2ch4-214	111	106	160	145	133	162
Launa el Pinar	1	PCH	2ch4-246	111	106	160	145	133	162
g40 × 1ch6–029	1	PEH	eh49	111	106	160	145	133	162
g15 × ch4–51	1	PEH	eh83	111	106	160	145	133	162
e2–31 × ch6–59	1	PEH	eh88	111	106	160	145	133	162
e1–102 × 1ch6–002	1	PEH	eh89	111	106	160	145	133	162
MPR	1	PCH	1ch4-057	111	106	160	145	133	162
MPR	1	PCH	1ch4-078	111	106	160	145	133	162
MPR	1	PCH	1ch4-089	111	106	160	145	133	162
Alamicamba	1	PCH	1ch4-245	111	106	160	145	133	162
Brus Lagoon	1	PCH	1ch4-249	111	106	160	145	133	162
Karawala	1	PCH	1ch4-258	111	106	160	145	133	162
MPR	1	PCH	1ch6-002	111	106	160	145	133	162
MPR	1	PCH	1ch6-029	111	106	160	145	133	162
2ee1–107 × 1ch4–089	1	PEH	bs6	111	106	160	145	133	162
2ee1–107 × 1ch4–089	1	PEH	bs7	111	106	160	145	133	162
2ee1–107 × 1ch4–089	1	PEH	bs9	111	106	160	145	133	162
MPR	2	PCH	1ch1-063	111	106	160	145	133	163
Slimla Sia	2	PCH	1ch4-247	111	106	160	145	133	163
2ee1–102 × 1ch1–063	2	PEH	eh51.1.16	111	106	160	145	133	163
2ee1–102 × 1ch1–063	2	PEH	eh51.1.17	111	106	160	145	133	163
e1–102 × ch1–63	2	PEH	eh90	111	106	160	145	133	163
MPR	3	PCH	1ch4-073	111	106	160	145	133	163
MPR	4	PCH	2ch4-203	111	106	160	145	133	162
Guanaja	5	PCH	1ch4-254	111	106	160	143	133	163
N Fla	6	PEE	1ee2-015	111	107	160	145	133	162
N Fla	6	PEE	1ee2-037	111	107	160	145	133	162
N Fla	6	PEE	1ee2-043	111	107	160	145	133	162
N Fla	6	PEE	1ee2-057	111	107	160	145	133	162
N Fla	6	PEE	1ee3-015	111	107	160	145	133	162
g5 × g20 N Fla	6	PEE	2ee1-105	111	107	160	145	133	162
g11 × g21 N Fla	6	PEE	2ee1-107	111	107	160	145	133	162
N Fla	6	PEE	1ee1–023	111	107	160	145	134	162
N Fla	7	PEE	1ee1–161	112	106	160	145	134	162
N Fla	8	PEE	1ee1–015	111	106	161	145	134	161
N Fla	9	PEE	1ee1–086	111	105	161	143	133	161
N Fla	10	PEE	1ee2–006	111	105	161	143	134	161
N Fla	10	PEE	1ee2–031	111	105	161	143	134	161
N Fla	10	PEE	1ee2–048	111	105	161	143	134	161
N Fla	10	PEE	1ee2–081	111	105	161	143	134	161

Table 1. (continued).

Provenance /Pedigree	Haplotype	Taxa	Genotype	Pt109567	Pt15169	Pt45002	Pt71963	Pt79951	Pt87268
N Fla	10	PEE	1ee3–005	111	105	161	143	134	161
N Fla	10	PEE	1ee3–027	111	105	161	143	134	161
N Fla	10	PEE	2ee1–088	111	105	161	143	134	161
eg × g 25 N Fla	10	PEE	2ee1–166	111	105	161	143	134	161
2ee1–107 × PEE	10	PEH	bs8	111	105	161	143	134	161
2ee1–102 × 2ee1–102	11	PEH	50.1.12	111	105	161	143	134	160
g23 × g26 N Fla	11	PEE	2ee1–102	111	105	161	143	134	160
N Fla	12	PEE	1ee1–142	110	105	161	143	134	161
N Fla	12	PEE	1ee1–074	110	105	161	143	134	161
N Fla	13	PEE	1ee2–012	110	105	161	143	135	161

[1] Region or provenance reported for trees in the reference population. Pedigree (or putative pedigree) reported for F_1 trees. For codes used in pedigrees see footnote 3. Incomplete pedigrees suffixed with an "?" indicate the two individuals where molecular data is inconsistent with crossing records.

[2] PEE = *P. elliottii* var. *elliottii*; PCH = *P. caribaea* var. *hondurensis*; PEH = PEE × PCH hybrid according to crossing records. PEH? = PEE by unknown parent on the basis of nuclear microsatellite data (data not shown).

[3] Genotype:- 1st digit refers to selection generation, ee= *P. elliottii* var. *elliottii*, ch= *P. caribaea* var. *caribaea*, 2nd digit is the region designation in Queensland from where selection was made, Number following dash is the selection number.

[4] Haplotypes reported as length of PCR product in base pairs as determined by ABI 310 analysis of 5` dye labelled primers. These sizes do not necessarily equate to the actual fragment size.

Multiplex amplification of chloroplast microsatellite loci

Multiplex PCR was carried out using the 6 polymorphic loci with 5' dye labelled forward primers (see list above). Reactions were carried out using 20 ng of DNA template in a total volume of 20 ml in a buffer with the final concentrations of; 1× PCR buffer (Life Technologies); 0.8 mM dNTPs (total), 1U of Platinum Taq (Life Technologies), 2.0 mM $MgCl_2$ (Life Technologies) and 0.1 mM of each of primer. PCR products were separated using capillary electrophoresis as described above except; PCR product was initially diluted 1:10, the size standard was TAMRA 500 (Applied Biosystems) and the collection settings used Module C. After initial testing, primer concentrations were adjusted to achieve more uniform peak heights amongst individual PCR fragments. Peak heights were influenced by the efficiency of PCR for each locus, the signal strength of each dye and the properties of the individual primer synthesis and labelling reaction. The final concentration of primer-pairs was adjusted to 0.1 mM for Pt 45002, Pt79136, Pt79951 and Pt87268, 0.2 mM for Pt109567 and 0.05 mM for Pt15169F-Tet primer.

DNA sequencing to characterise interspecific polymorphism

A PEE and a PCH individual (2pee2-081 and 1pch6-002) were sequenced for each of the six polymorphic loci to establish the basis of sequence variation between the taxa. Direct sequencing was carried out on the PCR fragments amplified according to (VENDRAMIN *et al.* 1996) with the following exceptions. Reactions were carried out in 50 ml volumes using 20 ng of total genomic DNA template. Taq polymerase and PCR buffer was supplied by Roche Applied Science (Mannheim, Germany). PCR products were purified using purification columns (QiaQuick PCR, Qiagen) and quantified by comparison with molecular weight standards on an 2% agarose gel. Approximately 10 ng of template was used in forward and reverse sequencing reactions with the appropriate PCR primer (Big Dye Terminator Chemistry (Applied Biosystems)). Sequencing reactions and gel separations were carried out at Newcastle DNA (Newcastle, Australia). Forward and reverse sequence were aligned using ClustalW algorithms (THOMPSON *et al.* 1994) within the Bionavigator suite of programs (ANGIS, Canberra, Australia).

Scoring and data analysis

Single locus haplotypes (SLH) were determined for each of the 64 samples. Those loci which were polymorphic within the reference population were jointly analysed to form multi-locus haplotypes (MLH) for each sample. Unbiased haplotype diversity measures were calculated as per (VENDRAMIN *et al.* 1998).

RESULTS

Diversity in pine chloroplast microsatellites within the reference population and distinction of the PEE and PCH taxa

Polymorphism within the reference population was found at six out of the 10 loci investigated. The haplotype size for the four non-polymorphic loci as determined by 5' dye labelled products on the AB 310 were 136bp, 109bp, 125bp and 142bp for Pt30204 Pt26081 Pt102584 Pt36480, respectively. At the polymorphic loci, the number of haplotypes ranged from 2-4 (Table 1). Haplotypes differed by increments of a single base pair. These haplotypes were readily and repeatably resolved using capillary electrophoresis (data not shown). Haplotypes based on fluorescently labelled dUTP products had bimodal electoropheogram peaks, as both stands of PCR product were labelled, whereas 5' dye labelled products were represented by a single peak. Haplotypes were readily distinguishable by both labelling systems however. Sequencing of a representative from each of the PEE and PCH taxons for five out of six of the polymorphic loci indicated the basis of the polymorphism was expansion or contraction in the mono-nucleotide repeat sequence (data not shown). With the exception of 163bp haplotype for Pt87268, all single locus haplotypes found within the PCH taxon were also found within PEE, however, PEE also had unique haplotypes.

By jointly analysing SLH, 13 MLH were identified within the reference population. All PCH representatives (MLH1-5) were distinguishable from PEE representatives (MLH6-13). Multilocus haplotype 1 was the most common, with the majority of PCH individuals (83 %) possessing this haplotype, a further 7 % of PCH possessed MLH 2 whereas the remaining 10 % (3 individuals) were unique, representing MLH 3,4 and 5 (Table 2). There were two common MLH in PEE, MLH 6 and 10, where the majority (5/6) of the remaining MLH were uniquely represented. Consequently, the haplotypic diversity based on MLH for each taxa was higher for PEE (0.7) than PCH (0.3) (Table 2).

Verifying F_1 hybrids between PEE and PCH

Paternity of 12 putative F_1 hybrids was evaluated using MLH assays. The MLH of all putative F_1 hybrids, except two previously suspected of resulting from fertilisation by a non-intended parent (see below), were PCH unique haplotypes (*i.e.* MLH 1 or 2). The MLH of the F_1 were also consistent with the MLH of the documented pollen donors in all cases (8/10) where the pollen donor haplotype was determined (haplotypes for two pollen donors, ch4-51 and ch6-59 were not determined as these trees were no longer available). For example, 1ch1-063 was the pollen parent of eh51.1.16, eh51.1.17 and eh90. 1ch4-089 was the pollen parent of BS6, 7 and 9 and 1ch6-029 was the pollen parent of eh49. As these F_1 all possessed PCH haplotypes it was possible to confirm that they

Table 2. Multi/locus haplotype diversity and frequencies for three taxa, *P. elliottii* (PEE), *P. caribaea* (PCH) and their F_1 hybrids (PEH) (nb diversity index was not calculated for PEH taxon).

Taxon		MLH Haplotype No[1]													Sums	Diversity[2]
		1	2	3	4	5	6	7	8	9	10	11	12	13		
PEE	Count						8	1	1	1	8	1	2	1	23	0.70
	Freq.						0.35	0.04	0.04	0.04	0.35	0.04	0.09	0.04	1	
PCH	Count	24	2	1	1	1									29	0.29
	Freq.	0.83	0.07	0.03	0.03	0.03									1	
PEH	Count	7	3												10	
	Freq.	0.7	0.3												1	

[1] See Table 1 for description of MLH

[2] Unbiased haplotype diversity - see VENDRAMIN) *et al.* (1998).

were all hybrids and had not resulted from a self-fertilisation of the PEE mother or from an outcross to another PEE.

Two putative F_1, BS8 and 51.1.12, had previously been suspected as resulting from a cross with an unintended pollen donor on the basis of nuclear microsatellite markers (data not shown). The sample BS8 possessed MLH 10, a common PEE haplotype that was different to its known seed parent, 2ee1-107 (MLH 6), suggesting this tree resulted from a pollen contaminant from another PEE. The sample 50.1.12 possessed a PEE haplotype MLH 11 which was the same as its known seed parent 2ee1-102. This data was consistent with previous data from nuclear microsatellites which suggested it was a product of a self fertilisation (SHEPHERD *et al.* 2002). This tree had been produced from a controlled pollination where PCH pollen had been mixed with heat sterilised seed-tree pollen to try and improve fertilisation rates (M Dieters pers. comm.).

DISCUSSION

We found that combining haplotypes from six *Pinus* spp. chloroplast microsatellite loci provided sufficient resolution to assign all representatives in our PEE and PCH reference populations to their respective taxon. Furthermore, because the chloroplast locus in *Pinus* spp. is paternally inherited, we confirmed interspecific hybridisation and paternity of putative F_1, as well as distinguishing probable "selfed" progeny and a putative PEE outcross offspring. As the reference populations represents about 5–10 % of the breeding populations, our results suggests the power of the assay to discriminate members of the two breeding populations should be extensive, but deeper sampling will be required to confirm this. Our data suggest that, more intensive sampling will be necessary in PEE compared to PCH because of the greater haplotype diversity of this taxon. If necessary, it should be straightforward to increase resolution by the addition of further chloroplast microsatellites into the assay. PCR assays such as this are largely amenable to automation and suited to high throughput applications. The robustness of this assay based on chloroplast loci allowed multiplexing of loci, which can be problematic for nuclear markers. The advantage of a multiplex PCR in this case was estimated to give a 75 % savings in cost and time required for data generation above that of single locus assays (data not shown).

The MLH assay should find applications in the breeding and production of hybrid pine where large-scale quality control testing is required to confirm hybrid status. For example, in the QFRI clonal forestry program, the assay could be applied at strategic points in the production chain. Low performing non-hybrids could be eliminated from production hedges before they undergo high rates of multiplication and prior to the availability of field testing data to base selections upon, thereby saving nursery costs. The assay could also have a role in the design and monitoring of seed orchards. By haplotyping candidate parents and selection of parents planted in a seed orchard, it would be possible to monitor the level of selfing as well as outcrossing to non-target pollen donors. The assay could also have a role in the identification of artificial and natural hybrids. Chloroplast microsatellite markers have been found to work broadly across the major sections of pines e.g. (BUCCI *et al.* 1998; VENDRAMIN *et al.* 1998; VENDRAMIN *et al.* 2000; VENDRAMIN *et al.* 1996). Artificial hybridisation is successful amongst most combinations of the four commercial species in the *Australes* subsection: shortleaf (*P. echinata* Mill.); loblolly pine (*P. taeda* L.); longleaf (*P. palustris* Mill.); and slash pine (*P. elliottii* Engelm.) and natural hybridisation is also prevalent amongst this group (WAGNER *et al.* 1992).

The high diversity in PEE compared with PCH was unexpected given the narrower geographic range from which PEE was sourced. The high diversity in PEE, however, was consistent with previous molecular studies of the chloroplast (NELSON *et al.* 1994; SHEPHERD & HENRY 2003; WAGNER *et al.* 1987). The high diversity in PEE may reflect past introgression of *P. caribaea* into this species (NIKLES 1966).

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