PARENTAL RECONSTRUCTION FOR BREEDING, DEPLOYMENT, AND SEED-ORCHARD MANAGEMENT OF *EUCALYPTUS NITENS*

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ABSTRACT

A new open-pollinated breeding strategy for *Eucalyptus nitens* (Deane & Maiden) Maiden in New Zealand was explored using microsatellite markers to reveal the parental identity of forwards selections. Microsatellites are the preferred markers to reveal genetic relationships between individuals, largely owing to their co-dominant inheritance.

Forwards selection of individuals for the breeding population and future deployment was simulated using 10 open-pollinated seedling offspring from each of 10 clones in a clonal seed orchard. A set of 15 microsatellite markers was chosen from the 41 initially tested. Ninety of the 100 progeny sampled matched consistently to a single mother and father and 13 of these were evidently selfs. Eight had a maternal match only; this would indicate that either there was contamination by pollen from outside the orchard or there was an occasional failure by the marker set to identify the orchard pollen. One seedling had no maternal match and it was not possible to discriminate between two fathers in another.

There was a broad level of outcrossing at the individual and provenance levels, and there appears to be little indication that individual clones favour specific pollens. Estimates of the coefficient of inbreeding and coefficient of co-ancestry were derived for the seed orchard and forward selections.

Keywords: parental reconstruction; breeding strategies; *Eucalyptus nitens*.

INTRODUCTION

The area of *Eucalyptus nitens* planted in New Zealand each year is only about 1000 ha, and so a highly cost-efficient breeding strategy is required to capture genetic gain from the breeding programme. The breeding population of *E.nitens* is

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represented by trials of 300 families of first-generation open-pollinated progeny, established in the North Island in 1990. The breeding strategy used two trial designs: open-pollinated single-tree-plots for breeding-value estimation, and forwards-selection blocks where families were planted as row-plots and divided into disconnected sublines. Within-family selection in the forwards-selection blocks was intended to identify individuals as parents for the next generation and for the establishment of clonal seed orchards (Cannon & Shelbourne 1991). Unfortunately, the sites of these progeny tests did not promote good flowering and a different strategy for moving the breeding population forwards was required. The high cost associated with controlled pollination to produce full-sib progenies could not be justified for this species. Therefore a strategy was explored that was based on open pollination, but with the benefits of controlled-pollination.

The best individuals from the 30 top-ranked families were forwards selected at age 5 years, grafted, and established as clonal seed orchards for the production of improved seed. A larger group of selections from 180 families were also grafted and planted on good-flowering sites as breeding archives. The intention was to collect open-pollinated seed from the seed orchards and clonal archives, to plant these seedlings in family tests, and to later select new orchard parents and a breeding population using molecular markers to identify male parentage of selections.

Groups in various countries are developing dynamic molecular biology research projects with forest trees, involving mainly pines, poplars, and eucalypts. Reviews of the status of some of these efforts published recently (Jain & Minocha 2000) concluded that the most important current application of genetic markers in tree improvement is for verification of identity and pedigrees of genotypes. Genetic markers can also assist with seed orchard management in estimation of the levels of pollen contamination, estimating selfing rates and inbreeding, determining mating patterns within the orchard, and determining the effects of orchard management practices such as spacing, of location within the crown of seed collection, and of gene flow (Moriguchi *et al*. 2004; Hansen & Kjær 2006).

Microsatellite markers have given the most robust results for genotyping analysis. Paternity testing of superior selections derived from hybrid crossing has been used in Brazil and elsewhere, using a battery of microsatellite markers (Grattapaglia *et al*. 2004); Random Applied Polymorphic DNA (RAPD) markers are used extensively for identifying clones in seed orchards (De Laia *et al*. 2000), testing the effectiveness of supplementary pollination techniques (Patterson *et al*. 2004). Molecular characterisation of stands using microsatellite markers has been incorporated into several breeding programmes (Lefort *et al.* 1998). Markers have also been used to confirm the misidentification of individual trees in genetic experiments (Vaillancourt *et al.* 1998) and for enhancing seed-orchard management techniques (Seido *et al.* 1999). Lambeth *et al.* (2001) reported the first use of parental analysis using a

polymix progeny as an alternative to full-sib breeding with *Pinus taeda* L., thus reducing costs with minimal sacrifice of genetic gain.

Given the previous strategy of open-pollinated single-tree-plots and forwards-selection blocks, parental reconstruction of open-pollinated progeny could be used to deliver general combining ability (GCA) estimations and forwards selections from a single field test. A study was set up to determine the feasibility of this strategy to advance the breeding population whilst securing pedigree identification of forwards selections.

A set of microsatellite markers needed to be developed that would identify individual genotypes of the *E. nitens* breeding population. Then the efficacy of that marker set to verify or accurately identify both the maternal and paternal parents could be tested. The procedures and results of that study are reported here and the potential for applying this technology to the development of an efficient breeding strategy for *E. nitens* is discussed.

MATERIAL

The material used in this study came from a clonal seed-orchard that was established in 1998 in Southland (latitude 46°35´S, longitude 168°56´E) by Southland Plantation Forests of NZ Ltd, known as Tinkers Clonal Seed Orchard. The clones in the orchard were forwards-selected from an open-pollinated progeny trial of 300 families based on an index including diameter growth, stem form, branching habit, and basic density, all measured at age 5 years. Thirty trees, selected from 22 families, were propagated by grafting and a maximum of two individuals were selected from any family. The computer program "Noincest" (Low & Cannon 1994) was used to allocate clones to planting positions in the orchard (at 6 × 6 m spacing), ensuring that ramets of each clone were located as far apart as possible, with an average of 10 ramets planted per clone. The orchard was separated from other *E. nitens* plantings by at least 40 m planted with *E. regnans* F. Muell., or a minimum of 55 m of unplanted ground.

In 2004, 29 out of the 30 clones in the orchard were producing seed. Foliage from three ramets per clone was collected and used to obtain a consistent marker genotype of each of the 30 clones in the orchard. Thirty percent of all ramets planted in the orchard were tested and no misidentified ramets were found. Open-pollinated seed was then collected from the upper half of the crown, at two or more widely separated compass points around the crown, of each ramet. Seed from two or more ramets of each maternal-parent seedlot was sown individually in the nursery for later establishment as a second-generation progeny test.

Ten parent clones were chosen from among the 30 clones in the orchard. They originated from three populations in central Victoria — two from Rubicon, four

from Toorongo, and four from McAlister. In the nursery 10 open-pollinated seedlings from each of these 10 clones were randomly selected to simulate a progeny test within which plus-trees were to be selected. The resulting 100 samples (10 open-pollinated families × 10 seedlings) were coded and foliage was collected for parental identification.

Microsatellite Selection

This project did not involve the development of markers. Instead, existing markers were sourced for which sequence, size, and polymorphism information was readily available, typically in published form. A total of 41 well-characterised microsatellite markers used with various eucalypt species were selected for screening from a number of sources (Brondani *et al.* 1998, 2002; Bryne *et al.* 1996; CSIRO FFP 1996; Glaubitz *et al.* 2001; Grattapaglia *et al.* 2004; Van Der Nest *et al.* 2000). Markers were selected using the following criteria:

- In the public domain
- Identified in mapping studies and unlinked
- Mendelian inheritance
- Highly polymorphic (> four alleles)
- Robust, reproducible, and unambiguous
- Suitable for use in an automated sequencer
- Suitable for multiplexing (reducing the costs of polymerase chain reaction (PCR) and electrophoresis)

Markers with high polymorphism information content and exclusionary power, and a low frequency of nulls, were highly desirable. Markers were statistically analysed using the Cervus 2.0 software package. This software looks at allele frequencies, heterozygosity, null frequencies, polymorphism information content scores, and the exclusionary power for each marker. This assessment quickly revealed markers of poor quality. Allelic data were generated using the Applied Biosystems Genotyper® and GeneScan® Software programs. To ensure the integrity of the data, each genotyping was scrutinised by multiple readers. Data were exported to a database, providing a permanent electronic record that could be accessed and compared to other samples tested at any future date.

DNA Extraction and PCR Amplification

Genomic DNA was extracted from leaf material for all parent and offspring samples. The technique employed for this study is a reliable and widely used genomic DNA isolation method similar to that described by Stacey & Isaac (1994). Typically this is described as a CTAB method because the main component of the

buffer is a DNA-complexing detergent called CTAB (cetyltrimethyltetraammonium bromide). During optimisation, several improvements to the method were instigated. The most successful was the inclusion of a bead-milling procedure for homogenisation of the leaf material. Bead milling uses a large number of minute glass or ceramic beads that are vigorously agitated by shaking or stirring. Cell integrity is compromised due to the crushing action of the ceramic beads as they collide with the cells. Moreover, this step is performed in the presence of buffers containing a detergent (in this case CTAB) to break down/emulsify the lipid bi-layer structure of the cell membrane (and nuclear membrane), causing the lipids and proteins to precipitate and allowing the cell contents to spill out into the solution.

Each microsatellite marker consists of a set of oligonucleotide primers that are designed to target specific repeat regions from the genomic DNA, i.e., each nucleotide primer sequence is complementary to the regions immediately flanking the repeat. During PCR these repeat regions are greatly amplified, increasing in abundance by orders of magnitude, thus permitting detection by the instrument. The number of repeats at a given locus directly influences the size of the PCR product. Electrophoresis allows these sizes to be determined, facilitating the assignment of alleles and genotypes for a specific locus. Using multiple loci produces a DNA profile that can be used for comparison and from which parentage can be assessed. Related individuals will have alleles of the same size in common, whereas unrelated individuals may have alleles of different sizes at any given locus. For this study, detection and electrophoresis were performed using the ABI PRISM® 3100 Genetic Analyzer from Applied Biosytems. Amplified products were labelled with fluorescent dyes that emit a fluorescent signal when excited by laser. Applied Biosytems Data Collection® Software converted the electronic signals into visual images that can be analysed by the user.

Identical PCR conditions were used to trial all 41 markers. Standardising the conditions improved efficiency and allowed multiple markers to be amplified in a single reaction (multiplexing).

RESULTS AND DISCUSSION

Identification of Co-dominant Microsatellites, and Parental Identification

Of the 41 markers trialed, 15 were selected for use in this study (Table 1).

Paternity determination was carried out on the 100 seedlings using a sequential paternity exclusion procedure (>99%) and based on the data management system Corel Paradox 8. Exclusion was declared when the paternal allele in the progeny sample was not present in the candidate parent clone for at least two independent

TABLE 1–Publicly available microsatellite markers used for the parental reconstruction of open-pollinated progenies.

Primer name	Actual size (pig tails)	Dye	PIC*	Forward	Reverse	Observed hetero-zygosity	No. alleles
EMBRA10	116-148	VIC	0.814	GTAAAGACATAGTGAAGACATTCC	AGACAGTACGTTCTCTAGCTC	0.804	13
En6	88-107	NED	0.836	GAGCTGGAAATGGAGCAGAC	TCAATTTTTGCCTCTCCCC	0.826	13
EMBRA64	256-266	PET	0.416	CAGAACCCAGCGGAGGA	AGCTCCCTTCACAAGGTA	0.5	5
Es054	102-118	FAM	0.5	GGAAGAAATCAAACTGGACACC	TTTGCGACTACCATTTTCACC	0.522	9
Es140	117-151	NED	0.794	GCTCATTGTACTGCACAGAGG	AAGGCACCAACAGTACCTGG	0.778	12
Es211	90-103	VIC	0.509	GGGAGAGCTGATTGAGTAATTG	GCTGAGAATGGAAGCACATC	0.587	5
FRMSA3	165-199	FAM	0.665	TTATGGAAGAGAAAGACCAGCC	TTCGTCCGCGAATAGAAT	0.733	6
FRMSA4	308-320	VIC	0.415	GACGATGAAGATGAGGATGG	GCAACAGCGAAACTGAAAAT	0.565	4
EMBRA39	128-152	PET	0.613	GCATTCGTACTCATTTTCAA	GCATCGAGAGTGGATTAGTT	0.622	5
EMBRA63	182-230	PET	0.871	CATCTGGAGATCGAGGAA	GAGAGAAGGATCATGCCA	0.891	16
Eg126	344-384	NED	0.88	GAGGTCGAACGCAAGATAGC	TCTTATGGGGACATCAAGCC	0.957	14
Eg98		VIC	0.77	GCGAAGAAGCCTGTGATTTC	TGGGATCATCCGAAAAGATG	0.783	9
Eg99	184-202	NED	0.497	CTCATCAGCCTCCGAAACAC	GAAAGGAGGGACCTTTGAGG	0.568	8
Eg61	315-373	PET	0.877	AAAACGAACCACCCTTCCTC	CCTTTTGATGGGACTTGGTG	0.87	16
Eg65	244-279	VIC	0.561	CGGCCTCATTTCTCTAGGTG	GGCTAGACTAGGGGAAAGCG	0.556	9
						Average	9.6

* Polymorphism information content

markers, to avoid false exclusions due to mutation or null alleles. The statistical probabilities were not calculated, given that the sample was deemed inadequate for obtaining good estimates of allele frequencies; each progeny and parent comparison was reported as either consistent or not.

The marker set was able to resolve parentage to a single two-parent combination for 90 of the 100 progeny (including 13 selfs). A further eight seedlings were matched to a maternal parent only. This would indicate that either there was contamination by pollen from outside the orchard or there was a failure by the marker set to identify the orchard parent. One seedling had no maternal match and another had a maternal match with multiple possible fathers.

Previous studies have demonstrated that two general types of error occur at non-negligible rates, even when highly variable markers are used (Oddou-Muratorio *et al.* 2005). Firstly, a false paternity can be assigned, and secondly, there may be a failure to assign paternity when the candidate father is present in the paternal dataset. However, in this study, the lack of multiple matches in the parental reconstruction data attests to the high exclusionary power of this 15-marker set (>99%). A total paternal exclusion probability would give an illusory ability to assign paternity based on a given set of markers (Chakraborty *et al.* 1988). The assignment of 30 maternal and paternal parents to 100 progeny is a stringent but somewhat exaggerated test and suggests that this marker set is robust and would provide excellent resolution for parentage at more practical levels. Currently, the 15 markers exist in a format suitable for routine testing, but further optimisation should reduce the costs of PCR and electrophoresis.

Additional markers would not have translated into a higher number of single matches for this data set. But in situations where a large number of putative parents are closely related and/or where large numbers of offspring are matched to multiple parents additional markers would presumably be beneficial.

The 15 markers selected originated from a range of eucalypt species and should demonstrate some cross-species utility. The application of this marker set to other eucalypt species is currently being tested with *E. fastigata* Deane & Maiden. It is likely that a smaller, secondary set of three to four species-specific markers would be required as a supplement for each new eucalypt species investigated.

Seed Orchard Management Issues

Inter-provenance crossing

A concern during the establishment of the clonal seed orchard was the uncertainty as to whether cross-pollination could occur among all clones. The clones originated from a mix of the three provenances — Toorongo, McAlister, and Rubicon — and it was not certain that the flowering times among the clones from different

provenances would coincide. This study has shown that substantial crossing occurs between provenances. Among the 90 samples that had both the mother and father identified by the microsatellite marker set, 56.7% were inter-provenance crosses. This result suggests that flower synchrony among the provenance groups is generally high and supports the mixed-provenance orchard design. Despite the small number of clones in the orchard from Rubicon (Table 2) this provenance is present in the progenies as a pollen parent at very close to the overall expected rate. This level of outcrossing between provenance groups (Table 2) is likely to increase the genetic gain of the orchard seed collections.

TABLE 2–Number of provenance/inter-provenance crosses among the 90 seedlings with maternal and paternal identification. In brackets is the number of clones contributing as mothers and fathers

Provenance of mothers	Provenance of fathers		
	McAlister (8)	Toorongo (20)	Rubicon (2)
McAlister (4)	18	17	3
Toorongo (4)	12	20	2
Rubicon (2)	6	11	1

Important benefits of this research lie in opportunities for improved seed orchard management (Ericsson 1999; Goto *et al*. 2001; Gemas *et al*. 2004). Some advantage will be accumulated in each generation by incorporating pedigree reconstruction in the forwards selection. Pollination dynamics within the orchard can be monitored, and levels of pollen contamination may also be detected and expected genetic gains adjusted.

Parental reconstruction will allow the thinning of clones from the orchard, based on the knowledge of their pollen contribution. Inbreeding levels can be monitored and orchards designed to optimise desired crosses. Deployment of seed by individual seed parents, and forwards selecting of individuals from these plantings, would enhance within-family selection.

Among the 10 seedlings from each of the 10 clones used in this study there was a broad representation of different fathers. For example, from the sample of 10 seedlings of Clone 3, all were successfully matched to a mother and a father (Table 3). Among these 10 seedlings there were eight different pollen parents contributing to the offspring. This shows that pollination was occurring across a large number of clones in the orchard, not just the immediate neighbouring ramet, and there appears to be little indication that individual clones favour specific pollens. There were 30 clones planted in the orchard and 26 of those clones were

TABLE 3–Number of fathers per clone among the open-pollinated seedlings with full parental reconstruction

Clone	1	2	3	4	5	6	7	8	9	10
No. seedlings with full parental reconstruction	9	9	10	9	10	10	9	7	8	9
No. fathers	6	5	8	9	7	7	8	4	7	6

represented as male parents in the 100 seedlings sampled. In other words, 19 (86%) of the 22 families in the orchard contributed as males to the 100 seedlings sampled. This clearly shows that the orchard is effectively producing a high level of outcrossing among the genotypes planted and the intercrossing will mean some enhancement of the genetic gain in the orchard seed.

Pollen isolation

Eucalyptus nitens is insect-pollinated and the most commonly quoted figure for the mean effective pollination distance is 42 m (Sedgley & Griffin 1989; Moncur & Kleinschmidt 1992). This defines the width of the buffer zone recommended to prevent contamination within the seed orchard from neighbouring trees. The movement of insects within the crowns of individual trees and between trees has also been the subject of study. This information assists with decisions on the layout of clones within the orchard, in order to maximise outcrossing. Patterson *et al.* (2001) concluded that seed collectors should confine collections to the mid- to upper-third of the crown to ensure acceptable levels of outcrossing in the seed. The seed collected from the ramets in Tinkers Seed Orchard was collected from the upper crown and from two or more points around the tree. Using the marker set and fingerprint data for the clones in the orchard, it would now be possible to re-collect seed from the lower crown and determine the comparative outcrossing levels. Seed-orchard managers would certainly prefer to collect from more easily accessible lower branches and reduce collection costs, if adequate outcrossing is shown to occur in the seedlots.

Status number, coefficient of inbreeding, and co-ancestry

The level of outcrossing among parents can be determined using microsatellite markers and parental analysis. The correct coefficient of relationship can be applied to calculate heritability more precisely, and breeding values of breeding population and seed orchard parents can thus be more accurately predicted (Gea *et al.* 1997). Coefficients of relationship and status number in this study were derived using an algorithm developed by L.Gea, P.A.Jefferson, and S.Weaver (Lindgren *et al.* 1997).

Ritland (1996) showed that a set of eight marker loci with an average of 12.9 alleles per locus, each with 10 informative alleles, would be needed for adequate estimation of relatedness. The set of 15 markers used in this study had an average of 9.6 alleles per locus. If the number of alleles per locus were distributed with roughly equal frequencies (Kumar & Richardson 2005), the precision of estimates of relatedness could be improved. Nevertheless, 9.6 alleles per locus gave acceptable estimates of coefficient of inbreeding ($f = 0.054$), and coefficient of co-ancestry ($\mathbf{F} = 0.034$), for the forwards-selected sample (Table 4).

TABLE 4–Coefficient of co-ancestry, coefficient of inbreeding, census, and status number for Tinkers Seed Orchard, and randomly forwards-selected sample of open-pollinated progeny.

	Census number	Status number	Coefficient of co-ancestry **F**	Coefficient of inbreeding f
Tinkers Seed Orchard	30	23.4	0.021	0.00
Forwards-selected sample	100	14.7	0.034	0.054

Future Management of *E. nitens* Breeding Population

Customarily, the breeding values of selections are obtained by testing their progeny. The best genotypes for use in a seed orchard are identified either by backwards selection of parents or by forwards selection of the best individuals within the better progenies. The selection intensity among the individual offspring of a family is restricted by the number of individuals per family that can be included in a trial, usually approx. 30.

In future, second-generation progeny trials will be established with open-pollinated progenies collected from breeding archives and/or orchards. Pedigree reconstruction of the forwards-selections from these progeny trials would provide the full pedigree of parents for future seed orchards and breeding-population. The use of parental reconstruction in open-pollinated trials would require only a single trial (but multiple sites) for both parental GCA estimation and forwards selection.

For the third generation, the maternal identity will be certain, but paternal identity will be limited to grandparent information unless the whole progeny test is genotyped. Selected individuals could again be grafted and established in a new clonal archive where open-pollinated seed could be collected, as for the previous generation. Parental identity and number of related parents tested per site can be defined and related parents can be allocated to different progeny tests, effectively sublining the breeding population. That would enable paternal contributions of future forwards selections to be managed and largely pre-determined. Long-term management of the breeding population could require clonal archiving every second generation to reinstate full pedigree identity.

Potential problems of parental exclusion failures with a large breeding population can be mitigated by sublining. The effects of various rates of parental exclusion on expected genetic gain and decline in status number could be modelled. Lambeth *et al.* (2001) and Kumar & Richardson (2005) suggested that the value of microsatellite markers for determining paternity is limited and they should be used cautiously because alleles that are identical-by-state may not necessarily be identical-by-descent. Lambeth *et al.* (2001) asserted that for conifers it would not be possible to assign the paternity of progeny generated from a complete mix of individuals if there was relatedness in the population. In our study this was not the case; the full parentage was identified for 90% of the open-pollinated seedlings tested where the parents derived from an orchard with a coefficient of co-ancestry of F=0.021 (Table 3).

The costs of genotyping a high proportion of the breeding population could be prohibitive, given the limited scale of the planting programme. Reducing the costs of collecting, processing, and genotyping samples and marker analysis has been achieved largely by sampling within a single orchard and/or clonal archive. In principle, it would be possible to select on the basis of pollen-parent as well as seed-parent GCA, in addition to within full-sib family information. However, further logistical refinements will be needed in order to make this technique cost-efficient.

Genetic Diversity Management

Measuring genetic diversity by status number from seed collected in an orchard has been suggested by Kang *et al.* (2002). The formulae consider number of seeds, ratio of selfs, fertility of pollen parents, relatedness among pollen parents, and the amount of pollination contamination from outside the orchard. In this study, the coefficient of inbreeding, coefficient of co-ancestry, and status number calculated from open-pollinated seedlings derived from a seed orchard were recorded (Table 4). The use of microsatellite markers to reconstruct the parentage of open-pollinated offspring is a proactive approach towards biodiversity management, as well as determining pollen contamination (even from genetically modified organisms) within an open-pollinated orchard. Seedlots could be certified with more quantitative measures of biodiversity number that would enhance the stand value from a biodiversity perspective.

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