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Parental reconstruction in breeding programmes: An efficient approach for breeding, deployment and orchard management for *Eucalyptus nitens*

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EXECUTIVE SUMMARY

Microsatellites (SSR) are ideal markers to reveal genetic relationships between individuals owing to their co-dominant inheritance. Probably the most important current application of genetic markers in tree improvement is to address germplasm identification. Further to that, genetic markers assist with the seed orchard management in: the estimation of the levels of pollen contamination, estimating selfing rates and inbreeding, determining mating patterns within the orchard, and determining the effects of cultural practises – spacing, location within the crown of seed collection. However, in this study the primary objective was to determine if microsatellite markers could be used to redefine the efficiencies of an open-pollinated breeding strategy for *Eucalyptus nitens* in New Zealand.

A pilot trial with *E. nitens* explored the feasibility of reconstructing the parental identity of 10 open-pollinated offspring from each of 10 seed parents and applying this to the selection of plus trees in progeny tests. Eleven SSR markers from a total of 31 initially tested were eventually used. Eighty-two percent of the progeny samples matched consistently to a single mother and father and among these 10 were selfs. Six percent had a maternal match 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. Eleven percent had no maternal match and eight of those came from one particular clone, suggesting a null allele was present for one marker where a single maternal allele is not appearing in most of the progeny from this clone.

It therefore appears feasible to use parental reconstruction as a tool towards modifying the breeding strategy so as to improve the efficiency of the breeding cycle for *E. nitens*. Results from the study are also applicable in seed-orchard management and could also be used for implementing a biodiversity measurement for plantations of this species in New Zealand.

INTRODUCTION

A number of groups throughout the world are developing dynamic molecular biology research projects with forest trees, mainly involving pines, poplars and eucalypts. Reviews of the status of some of these efforts were published recently (Jain and Minocha 2000).

For fingerprinting analysis, SSR or microsatellite markers have given the most robust results. For reliable results a good estimate of the allele frequency has to be established. 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); RAPD markers are used extensively for identifying clones in seed orchards (De Laia *et al.* 2000), and molecular characterization 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 (McConnochie *et al.* 2002 and Ericsson 1999) and for enhancing seed orchard management techniques (Potts *et al.* 2000; Seido *et al.* 1999). Even with a much wider view of genetic processes than the ones used in this work, genomics will only succeed in contributing to the development of improved forests if it is strongly linked with intensive fieldwork and operational breeding.

The breeding strategy with *Eucalyptus nitens* in New Zealand required an efficient way to capture genetic gains from the breeding programme, fast generation turnover, and efficient delivery of genetic gains in the seed orchard.
First-generation open-pollinated progeny tests of *E. nitens* were established in the North Island in 1980 and 1990. The best individuals from the better families were forwards selected at age 6 years, grafted, and established as clonal archives and clonal seed orchards for the production of improved *E. nitens* seed. A larger group of families from these progeny tests were grafted and archives planted on good-flowering sites for breeding purposes.

A breeding strategy based on controlled pollination was rejected because of the relatively high costs of the breeding programme in relation to the limited area of *E. nitens* being planted. A breeding strategy that facilitated GCA estimation and forwards selection by parental reconstruction from a single trial, to offer cost savings and a shortened breeding cycle, was considered for the species. A pilot trial to explore the viability of this approach for advancing the breeding population using open-pollination is reported here.

Objectives of this study were to:

1) identify co-dominant microsatellites in *E. nitens*,
2) confirm clonal identity of seed- orchard ramets,
3) estimate the amount of pollen movement by insect pollination and pollen contamination from outside the clonal orchard,
4) estimate the percentage of inbreeding,
5) initiate a pilot study of parental reconstruction in an open-pollinated breeding strategy with *E. nitens,* and;
6) develop a model to predict seed quality in *E. nitens* seed orchards using simple measurements of reproductive parameters and verify it, using microsatellite markers and consequent paternity analysis. This model may allow improved prediction of breeding values of seed parents.

MATERIAL

The seed orchard used in this study was established in 1998 in Southland (latitude 46°35', longitude 168°56') by Southland Plantation Forests of NZ Ltd and is known as Tinkers Clonal Seed Orchard. The plus trees 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 at age five years. 30 selected trees were propagated by grafting and 22 families are represented with a maximum of two individuals selected from the top-ranked families. The computer programme 'Noincest' (Low and Cannon 1993) was used to allocate clones to a planting position in the orchard, to ensure that each replication of a clone was located a maximum distance apart. An average of 10 ramets per clone were planted at 6m x 6m spacing. The orchard is separated from other *E. nitens* plantings by at least 40m of *E. regnans* or a minimum of 55m.

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 prepare a consistent fingerprint of each of the 30 clones in the orchard. At the same time, open-pollinated seed was collected from the upper half of the crown of the same ramets and from two or more widely separated compass points around the crown. Each maternal-parent seedlot was sown individually in the nursery for later establishment as a second-generation progeny test.

Ten parent clones were selected from among the 30 clones in the orchard (2 Rubicon, 4 Toorongo, 4 McAlister provenance origin) Table 1, and 10 open-pollinated seedlings from each of these 10 families were randomly selected in the nursery to simulate plus-tree selection within a progeny test. The resulting 100 samples (10 open-pollinated families x 10 seedlings) were coded and foliage was collected for parental analysis.

Table 1: Provenance origins of the 10 selected clones used in the parental reconstruction test.

Clone No.	Provenance	Location
1	McAlister	Heyfield
2	McAlister	Macalister
3	McAlister	Mt. Skene
4	McAlister	Mt Shillinglaw
5	Rubicon	Blue Range
6	Rubicon	Blue Range
7	Toorongo	St Gwinear
8	Toorongo	Mississippi, Powellton
9	Toorongo	Mt Erica
10	Toorongo	Mt Toorongo Track

Microsatellite selection

A simple sequence repeat (SSR) microsatellite is a short, noncoding DNA sequence that is repeated many times in tandem within the genome. The repeated sequence is often very simple, consisting of two to four nucleotides, and can be repeated as many

as 100 times or as few as 10. For example the sequence for a microsatellite locus with a tri-nucleotide repeat may be "TAGTAGTAGTAGTAGTAGTAG...".
The number of repeats at a particular locus is hypervariable between individuals of the same species. For this reason, microsatellite sequences can be used for genetic fingerprinting and paternity testing.

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 a published article. The sources contributing directly to all markers trialed in this study are referenced in Appendix 1.

The following criteria were applied to select the SSR markers for this study:

1. The markers should be in the public domain.
2. Where possible, microsatellite loci that have been identified in mapping studies should be used, and those selected should preferably be known to be unlinked.
3. Microsatellite variants should be shown to exhibit Mendelian inheritance (highly mutable microsatellite loci may show departures from the Mendelian segregation, and would not be suitable for genetic distance analysis).
4. Sufficient polymorphism seen (greater than 4 alleles).
5. Markers will undergo statistical analysis using the Cervus 2.0 software package. This software looks at allele frequencies, heterozygosity, null frequencies, PIC scores and the exclusionary power for each marker. This assessment quickly reveals markers of poor quality.
6. Markers should be robust and easily genotyped with clear, reproducible, unambiguous alleles. This is a major factor in deciding whether to keep or reject markers, as it directly influences the quality of the data.
7. Suitable for use in an automated sequencer.
8. Marker size ranges are compatible to allow multiplexing therefore reducing the costs of PCR and electrophoresis.

Out of the 31 markers trialed, nine failed to meet Criterion 6 and were accordingly failed outright, a further 11 markers demonstrated non-Mendelian segregation and were also removed. Thus 11 markers were used for the final analysis.

DNA extraction and PCR amplification

In general DNA extractions can be simplified to three basic and essential steps. Firstly the cell must be lysed (broken open) to release the nucleus. The nucleus (if present) must also be opened to release the DNA. At this point the DNA must be protected from enzymes that will degrade it, causing shearing. Once the DNA is released, it must then be precipitated in alcohol.

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 (1990). Typically this is described as a CTAB method because the principle 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.

After cell lysis the extract is usually centrifuged – all the heavy cell debris is pulled to the bottom of the tube, leaving DNA and other soluble molecules such as protein in the supernatant. The most commonly used method to remove the proteins is extraction with a mixture of phenol and chloroform. These organic solvents, which are immiscible with water, denature proteins and sequester them in the organic phase while the DNA remains in solution in the aqueous phase. The aqueous phase is then transferred to a clean tube where ethanol precipitation is carried out. The precipitated DNA forms a pellet in the bottom of the tube by centrifugation. The pellet is washed with 70% ethanol to remove most of the salt and any other small molecules that have precipitated with the DNA. The pure DNA can then be re-suspended in water or a suitable buffer for further manipulations such as PCR (polymerase chain reaction) amplification.

Each microsatellite marker constitutes a set of oligonucleotide primers that are designed to target specific repeat regions from the genomic DNA i.e. their nucleotide 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 was performed using the ABI PRISM® 3100 Genetic Analyzer from Applied Biosytems. Amplified products are labelled with fluorescent dyes that emit a fluorescent signal when excited by laser. Applied Biosytems Data Collection® Software converts the electronic signals into visual images that can be analysed by the user.

Identical PCR conditions were used to trial all 31 markers. Standardising the conditions improves efficiency and allows multiple markers to be amplified in a single reaction. This is ideal for routine testing on large sample sets because it reduces the cost of consumables and labour.

RESULTS

Fingerprinting

Foliage was sampled from three ramets per clone in order to produce a consistent fingerprint, resulting in the testing of 30% of all ramets planted in the orchard. There were no misidentified ramets found.

Paternity testing

A set of markers that can be amplified in the same PCR reaction and undergo electrophoresis in the same lane can be defined as a panel. The most efficient way to perform analysis is to have all markers working as a single panel and is the ideal format for medium to large scale testing. However this is not always feasible. Determination of the most appropriate number of panels to use depends entirely on the extent to which the sizes of the PCR products overlap and the ease with which alleles can be accurately identified. Take for example a parentage test utilising 12 markers, as a worst case scenario this test may require as many as 12 separate PCR reactions and 3 to 4 separate electrophoresis runs per sample. Clearly the labour and consumable costs involved are significantly increased over that of a single panel.

For this study the 11 markers that were selected for parentage analysis demonstrated a high degree of incompatibly in respect to their allele sizes. Consequently analysis of the current marker set must be performed in several panels for PCR and electrophoresis.

Allelic data was generated using the Applied Biosystems Genotyper® and GeneScan® Software programs. To ensure the integrity of the data, each genotype is scrutinised by multiple users. Data are exported to a database, providing a permanent electronic record that can be accessed and compared to other samples tested at any time in the future.

Paternity determination was carried out on the 100 seedlings using a sequential paternity exclusion procedure. This procedure was performed using custom-designed programs based on the database management system (DBMS) Corel Paradox 8, allowing for rapid and accurate analysis. Exclusion was declared when the paternal allele in the progeny tree was not present in the alleged parent tree for at least two independent markers, to avoid false exclusions due to mutation or null alleles.

Using the chosen 11 markers 82 of the 100 progeny were found to be consistent to a single maternal and paternal combination, and among these 10 were selfs. A further six 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. Eleven had no maternal match and eight of those came from one particular clone, suggesting a null allele was present for one marker where a single maternal allele is not appearing in most of the progeny.

Before the maternal information was included 12 markers were used in the analysis. Sixty-two of the progeny were matched to a single maternal and paternal combination. A further three percent were consistent with multiple pollen parents, while only one progeny sample was not matched to any pollen parent. The discrepancy was soon attributed to a single maker that was subsequently removed from the panel because it repeatedly demonstrated the presence of null alleles that were producing false

exclusions. This same marker had previously been suspected of contributing null alleles, but this was only proven with the inclusion of the maternal information. Note that the number of progeny matching to multiple maternal and paternal combinations did not increase with the reduction from 12 to 11 markers. The conclusion was that this marker set has excellent power to resolve parentage to a single two-parent parent combination.

Inter-provenance crossing

Among the 82 samples that had both the mother and father identified by the microsatellite marker set, 44 (53.8%) were interprovenance crosses. This result suggests that flower synchrony among the provenance groups is high, supporting the mixed-provenance orchard design. Despite the small number of clones in the orchard from Rubicon provenance (Table 2) it does participate in numerous offspring, at least as a seed parent.

Table 2. Number of provenance/inter-provenance crosses from 10 selected mothers (numbers of parents under consideration in brackets)

Provenance of mothers	Provenance of fathers		
	McAlister (8)	Toorongo (20)	Rubicon (2)
McAlister (4)	17		
Toorongo (4)	28	19	
Rubicon(2)	7	8	1

DISCUSSION

Microsatellite information and parental identification

The lack of multiple matches in the resulting parental reconstruction data is a testament to the high exclusionary power of this current 11-marker set. Additional markers would not have translated into a higher number of single matches for this data set. Where it would be beneficial is in situations where the putative parents are closely related and/or where large numbers of offspring are matching to multiple parents. Note there exists a sizeable body of markers left to evaluate should the need arise (Brondani *et al.* 2002). The assignment of 30 maternal and paternal parents to 100 progeny is a stringent but somewhat exaggerated test for any marker set. Extensive experience with developing marker sets for DNA parentage would suggest that this marker set is robust and should provide excellent resolution for parentage at more practical levels. Attaining single maternal and paternal matches for >95% of the offspring is entirely feasible.
Currently the 11 markers exist in a perfectly serviceable format for routine testing. However, further optimisation is recommended to reduce the costs of PCR and electrophoresis, particularly if large-scale projects are planned in the future. It is envisaged that significant improvements could be made with little difficulty, given some dedicated effort.
The current 11 markers originated from a range of a eucalypt species and should demonstrate some cross-species utility. The application of this marker set to other eucalypt species should be tested. It is likely that a smaller, secondary set of 3 to 4 species-specific markers would be required as a supplement for each new eucalypt species investigated.

Future management of *E. nitens* breeding population

Owing to lack of flowering in the forwards-selected plots (described in Cannon and Shelbourne 1991) selections were grafted in clonal archives. An analysis of the feasibility of moving the population forwards by controlled pollination was carried, out and it was concluded that although larger gains were to be expected when the selection was for seed orchards, for advancing the breeding population there were no real benefits. The breeding archive was then divided into an elite subgroup of top parents where CP crosses could be carried out to provide advantages of PAM (positive assortative mating).
The decision to advance the *E. nitens* breeding population by OP entailed an assumption that estimates of breeding values would be precise and at low cost. Orchards can be made more uniform in breeding values to compensate for lack of PAM without compromising status number, and flowering patterns can also be monitored.

Implications for modifying the breeding strategy and improving the efficiency of the breeding cycle are evident. Historically, the genetic value of a plus-tree selection is obtained by testing the progeny of the parent. The best genotypes can be captured for use in a seed orchard either by backwards selection to the parent or forwards selection of the best individual(s) amongst the progenies tested. The selection intensity among the progeny is restricted by the number of individuals per family that can be included in a trial, usually 30. A second trial, comprising forwards-selection blocks, is planted with larger numbers per family to increase the selection intensity. The use of parental analysis on OP trials would require only a single trial for both parental GCA estimation and more efficient forwards selection. Increased inbreeding can be mitigated by limiting the number of selected individuals per open-pollinated family. There would be no need

for a subline structure within the breeding population. The level of outcrossing among the parents can be determined using microsatellite markers and parental analysis. The correct coefficient of relationship can be applied to calculate heritability and accurately predict breeding values of the seed orchard parents.

Among 100 plus trees selections within an open-pollinated progeny test, it could be expected that the marker set could successfully reconstruct the parentage of a high percentage of the individuals.

Seed-orchard management

One of the important benefits of this research lies in the opportunities for improving seed orchard management. Thinning parents based on information that includes pollen contribution, monitoring inbreeding levels, and even designing orchards to optimise desired crosses, are among the tools that have become available by using parental reconstruction. Levels of pollen contamination may also be detected and genetic gains adjusted. Pollen isolation protocols could be redefined. Further applications of this technique will be the deployment of seed by individual seed parents. Forwards selection of individuals within these plantings will enormously enhance within-family selection intensity by increasing number of trees and opportunities for recombination.

A concern during the establishment of the seed orchards was the uncertainty about the cross-pollination that would occur between the families in the orchard, and the selections were from a mix of the three provenances, Toorongo, McAlister and Rubicon. Little information was available about the flowering times of the provenances at the orchard sites, or whether they would coincide. This study has shown that substantial crossing occurs between provenances. Among the 80 samples that had both mother and father identified by microsatellite marker set, 53.8% were interprovenance crosses. This level of outcrossing between provenance groups (Table 2) will likely increase the genetic gain of the orchard seed collections.

Among the 10 seedlings from each of the 10 clones used in this study there was a broad representation of different fathers (Table 3). For example, from the sample of 10 seedlings of Clone 5 nine seedlings were successfully matched to a mother and father. Among these nine seedlings there were 8 different pollen parents contributing to the offspring. This shows that pollination is 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 a specific pollen. There are 30 clones planted in the orchard, 25 of those clones were represented as male parents in the 100 seedlings sampled. And, 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.

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	10	2	9	9	10	9	8	7	9
No. Fathers	6	8	1	8	8	6	6	6	3	5

E. nitens is insect-pollinated and the most commonly quoted figure for the mean effective pollination distance is 42m, Sedgley and Griffin 1989, Moncur and Kleinschmidt 1992. This defined the width of buffer zone recommended to prevent contamination from neighbouring trees planted in plantations. The movement of insects within the crown of individual trees and between trees has also been the subject of study to assist with 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 recollect 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.

The availability of genetically improved open-pollinated *E. nitens* seed from clonal and seedling orchards has greatly increased in the last five years. When supply of seed exceeds demand the opportunity exists to maximise the gains from the seed orchard. This can be done in several ways. Collections can be made from a subset of maternal parents within the orchard that have been selected for a specific trait, e.g. high basic wood density. Roguing the orchard, removing clones that are lower ranked, will increase the genetic gain among the remaining families.

Biodiversity management

Measuring genetic diversity by status number, from seed collected from an orchard, has been suggested by Kang *et al.* (2001). The formulae considers number of seeds, ratio of seeds from selfing, fertility variation of pollen parents, relatedness among pollen parents and the amount of pollination by alien fathers outside the orchard. The Eucalypt Cooperative has the opportunity to apply the results of this study to take a proactive approach towards biodiversity management. Seedlots could be certified with a biodiversity number that would enhance the stand value from a biodiversity perspective – a reality not yet considered for *Pinus radiata* in New Zealand.

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APPENDIX 1.

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