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EUCALYPT COOPERATIVE

Report No. 6

Parentage reconstruction using microsatellite markers in *Eucalyptus nitens*: effect of crown position on pollination.

by
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Key words ***Eucalyptus nitens*, microsatellite markers, pollination**

SUMMARY

Parentage reconstruction using 16 selected microsatellite (SSR) markers was carried out on open-pollinated (OP) seedling families of *E.nitens*, grown from seed collected from a sample of 10 clones in a 30-clone clonal seed orchard. In the first study (Gea et al., 2007) seed was collected from the mid crown of the mother trees in the orchard, and in the second study (reported here), from the lower branches a year later. In both studies, genomic DNA was extracted from ten seedlings of each OP family.

In the first study, ninety of the 100 progeny sampled matched consistently to a single mother and father, and among these, 13 were evidently selfs. Eight had a maternal match only, one seedling had no maternal match and in another case discrimination was not possible between two fathers.
In the second study, the mothers indicated by the markers were all in agreement with the identity of the mothers in the clonal orchard. All progeny had paternal parents which matched the 30 possible clonal fathers in the orchard. The lower crown selfing rate was again 8%.

There were 30 clones planted in the orchard and 26 of those clones were represented as male parents in the 100 seedlings sampled in each study. This result and the free intercrossing among provenances shown, indicated that a high level of outcrossing was occuring among all the clones planted, in both the lower- and mid-zone of the crown.

INTRODUCTION

Eucalyptus nitens is grown on a limited scale in New Zealand for production of pulpwood. Its genetic improvement started in 1979 with the planting of open-pollinated seed from 80 parents of various provenances from the natural populations in central Victoria (Cannon & Shelbourne 1991, 1993). In 1990, in the second generation of selection, this breeding population was expanded to 310 open-pollinated families from parents chosen in both native populations and plantations in Australia and New Zealand. For reasons of economy and operational efficiency for improvement of what is only a minor species, the breeding strategy has utilised open- as opposed to control-pollinated progeny. Gea et al (2007) successfully developed the use of microsatellite markers (SSR) to reveal the parental identity of seedlings of open-pollinated families, collected within a clonal seed orchard of 30 clones. These clones were originally selected from the best 22 families, among 310 families planted in 1990. Reconstruction of male and female parentage was successfully carried out on 10 seedlings per clonal seed parent family of a sample of 10 clones from the orchard, using capsules collected in the mid-zone of the crown. The orchard offspring showed high levels of outcrossing and the selfing rate among the orchard clones was 8%. Seed collectors would prefer to collect from easily accessible lower branches to reduce collection costs, but there is a danger that this would increase the levels of selfing (Paterson et al., 2001). To test this assumption, in the study reported here capsules were collected from the lower branches of the same ramets of the same 10 clones as in the previous study, and seedling progeny characterised with SSR markers as before.

METHODS AND MATERIALS

Plant material

The seedlings of the 10 open-pollinated families studied derived from a clonal seed orchard, Tinkers Orchard in Southland, New Zealand, lat. 46°35'S, long. 168°56'E (see Gea et al. loc. cit.). The 30 clones in the orchard were grafted from ortets (trees) which were forwards-selected at age five years for diameter growth, stem form, branching habit and basic density from an open-pollinated progeny trial of 310 families. One or two individuals were selected from the 22 top-ranked families in the test. An average of 10 grafted ramets of each clone were computer-located in the orchard a maximum distance apart, using ‘Noincest’ software (Low and Cannon 1993). Separation from other *E. nitens* plantings was by at least 40m of planted *E. regnans* or 55m of open space.

Foliage from three ramets per clone was collected and used to obtain a consistent marker genotype of each of the clones in the orchard. This genotype database was used in the parentage analyses.

Open-pollinated seed was collected from the lower branches (versus upper branches in the previous study) from 10 clones in the 30-clone orchard. The same clones and indeed the same ramets in the orchard were collected from as in the previous study. Plants were then raised from which leaf material was collected from a random sample of 10 seedlings each, of 10 families. Genomic DNA was extracted from leaf material (see Gea et al. loc. cit.) for all offspring samples and multilocus genotypes were characterised at 16 SSR marker loci. This technique is a reliable and widely-used genomic DNA isolation method similar to that described by Stacey & Isaac (1994).

Cervus analyses

Parentage analyses were carried out using Cervus 2.0 (Marshall et al. 1998) and the marker genotype database for the 30 clones in the orchard. A two step procedure was used, first to test that progeny genotypes were consistent with the expected maternal parent and then, if maternal parentage was correct, to identify the most likely paternal parent.

The Cervus program cannot necessarily determine unambiguously the correct parentage for any individual progeny analysed. The ability to find the correct parents is influenced by:

- whether the correct parent(s) are in the list of candidate parents supplied
- the level of data scoring errors
- the frequencies of any “null” alleles
- the level of missing data in parental or progeny genotypes
- population frequencies of the progeny and parental alleles.

The Cervus output gives a list of possible pollen parents of each individual progeny seedling, along with their LOD scores, Delta values and a confidence level. A LOD score of zero implies that the candidate male parent is equally likely to be the true parent as is an arbitrary randomly-chosen individual. A positive LOD score implies that the candidate parent is more likely to be the true parent than an arbitrary randomly-chosen individual. The most likely candidate parent is the candidate parent with the highest (positive) LOD score. Delta is the difference in LOD scores between the most likely candidate pollen parent and the second most likely candidate parent. If the most likely

parent has a "*" confidence level then it has at least a 95% probability of being the correct parent.

RESULTS

Consistency of progeny genotypes with expected maternal genotypes

Multilocus genotype arrays were used to test each seedling progeny against all candidate (pollen and seed) parents planted in Tinkers Clonal Seed Orchard, with no prior expectations of parentage given to Cervus. Expected mothers appeared near the top of the list of possible parents (data not shown), with relatively high LOD scores. Data scoring errors, "null" alleles, missing data and the balance of rare versus common alleles can lead to situations where maternal parents, paternal parents and siblings of parents can all have high LOD scores. The expected maternal parentage was considered to be confirmed if the expected maternal parent was in the top four most likely parental genotypes and had a LOD score greater than 3. With these criteria, all the progeny of all 10 clones were consistent with being progeny of the expected mother.

Paternal genotype identification

The progeny data were reanalysed in Cervus with "known" maternal genotypes to determine the most likely paternal genotype (Appendix Table 1). From this analysis, selfs are identified if the maternal genotype is also the most likely paternal genotype, stray or "polluting" pollen from outside the set of candidate parents is detected if the most likely paternal parent has a LOD score less than zero, and in all other cases a most likely candidate parent is identified from within the orchard.

All 100 seedling progeny had a most-likely male parent, where Cervus gave a "*" confidence level. No stray pollen was detected and eight likely selfs were detected (8%). The apparent selfing rate ranged from zero (for 6 of 10 clones) to 40% for Clone 153.

A Cervus analysis includes a calculation of allele frequencies and an estimate (based on these frequencies) of the ability to exclude a randomly generated parental genotype from being the true parent of a randomly generated

progeny array. In this data set, Cervus estimates the failure to exclude a randomly generated parent from being the correct parent as less than 1 in 1000.

With the large number of highly variable markers used, as indicated by the heterozygosity values calculated by Cervus (0.681), it is to be expected that disagreements between progeny genotypes and expected maternal genotypes or the failure to find paternal parents from within the orchard, are most likely to be real and not the result of genotyping errors.

Posterior distribution for the proportion, p, of seedlings with parents identified, when 90 out of 100 are identified in a sample (as in the first study of Gea et al., 2007) is a Beta distribution:
(1) p ~ Beta (90.5,10.5)
This estimate assumes there is no difference or effect of the female parent and no errors with parents, incorrectly identified. This assumes a `non-informative' Beta (0.5,0.5) prior distribution for p.
The posterior distribution for p has a mean of 89.6%, mode 90.4% and 95% confidence limits are 83.0--94.7%.

For this study, posterior distribution for the proportion, p, of seedlings with parents identified, when 100 out of 100 are identified in a sample is:
(2) p ~ Beta(0.5,100.5)
In this case the posterior distribution for p has mean 99.5%, mode ca. 100% and the
95% confidence limits are 97.5--99.9%

Posterior distribution for the proportion, ps, of selfing, when 8 out of 100 plants sampled were determined to be selfed is:
(3) ps ~ Beta(8.5,92.5)
The posterior distribution for ps has a mean of 8.4%, mode 7.6% and 95% confidence limits are 3.9--14.5%.

DISCUSSION

The fathers of all 10 offspring of each of the 10 maternal clones sampled in the orchard were identified in this second study (Table 1), in contrast to the previous study where an average of only 9 out of 10 offspring per family had fathers identified. In this study, 16 SSR markers were used to identify the same female parents and the same possible male parents. The inclusion an additional marker (FRMA2) may explain the increased accuracy to identify parentage in this second study. The proportion of self-pollinated offspring was

8% in both studies, so there appeared to be no more selfing in seed from the lower crown than the mid crown. The seed collected for this study was collected a year later than for the first study, so there may have been some change in the pollen availability from different parents and in insect behaviour in that season.

There were 30 clones planted in the orchard and 26 of those clones were represented as male parents in the 100 seedlings sampled in each study. Two clones, 113 and 119, were not presented as pollen parents in either seed collection. Among the 10 seedlings from each of the 10 clones used in this study there was a broad representation of different fathers. This shows that pollination is occurring across a large number of clones in the orchard, not just the immediate neighbouring ramet, and within both the mid and lower crown.

Analysis of the provenance identity of all 100 seedlings (Table 2) shows that pollination has occurred freely amongst the three provenances, with the parentage of individual seedlings in all combinations of provenance and numbers of offspring in rough proportion to the numbers of fathers and mothers of each provenance. Therefore it appears that the timing of flowering of clones in the orchard is not provenance-dependent and that plenty of provenance crossing has occurred which will result in increased heterozygosity in orchard offspring and increased genetic diversity in future generations of the breeding programme.

These results provide independent confirmation of the results and conclusions of the first study (Gea et al. loc cit.). The present management of the breeding population of *E. nitens* has involved grafting and establishment of 180 parents in the breeding population in clonal archives on good flowering and seed producing sites. This will be followed by collection of open-pollinated seed and re-establishment of the breeding population progeny tests, later followed by forwards selection of new parents. It is now clear that SSR parentage analysis of forwards selections in these tests will be feasible and will allow rejection of selected trees which share the same fathers, thus controlling the development of relatedness in the breeding population. Provided balanced within-family selection is maintained in the breeding population, this strategy should be sustainable over several generations without serious increase in inbreeding levels.

The collection of seed from the lower crown does not seem to increase the levels of inbreed seed for *E.nitens*, as reported elsewhere for other species. Based on these results it is reasonable to collect seed from any or all parts of the crown of seed orchard grafts.

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Table 1. Number of seedlings with full parentage reconstruction per female parent, and number of fathers involved and selfs in each family, for first and second studies

Female Parent Clone No.	109	135	164	150	423	101	163	158	153	110	Totals
No. seedlings with full parental reconstruction	10	9	9	9	10	10	9	7	8	9	90%
	10	10	10	10	10	10	10	10	10	10	100%
No. of fathers	8	7	8	8	7	7	8	4	7	6	
	10	9	8	9	5	8	5	2	6	8	
No. selfs	2	1	0	1	3	0	0	0	1	0	8%
	1	1	0	2	0	0	0	0	4	0	8%

Seed from mid-crown – Study 1, first line. Seed from lower-crown – Study 2, second line.

Table 2. Number of crosses among 100 seedlings by provenance combination for both studies (number of mother and father clones by provenance, in brackets)

Provenance of mothers	Provenance of fathers		
	McAlister (8)	Toorongo (20)	Rubicon (2)
McAlister (4)	14	19	5
	15	24	1
Toorongo (4)	12	20	2
	19	21	-
Rubicon (2)	6	11	1
	8	9	3

Study 1, first line; Study 2, second line

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Appendix Table 1. *E. nitens* paternity results

Progeny ID	"Known" female parent	Most Likely male parent	LOD	Confidence	
101_1	002054N_77N_82N	002056N_86N_123N	14.1	*	
101_2	002054N_77N_82N	002N_41N_79N_100N	8.99	*	
101_3	002054N_77N_82N	002036N_39N_93N	11.3	*	
101_4	002054N_77N_82N	002064N_66N_74N_112N	10.6	*	
101_5	002054N_77N_82N	002040N_63N_105N	15.7	*	
101_6	002054N_77N_82N	002056N_86N_123N	0.063	*	
101_7	002054N_77N_82N	002057N_87N_111N	11.6	*	
101_8	002054N_77N_82N	002049N_101N_120N	2.16	*	
101_9	002054N_77N_82N	002038N_67N_94N	7.36	*	
101_10	002054N_77N_82N	002057N_87N_111N	16	*	
109_1	002050N_81N_96N	002071N	9.9	*	
109_2	002050N_81N_96N	002050N_81N_96N	9.69	*	**probable self**
109_3	002050N_81N_96N	002N_41N_79N_100N	12	*	
109_4	002050N_81N_96N	002073N_84N_89N	8.77	*	
109_5	002050N_81N_96N	002062N_68N_113N	12.9	*	
109_6	002050N_81N_96N	002116N_122N	14.6	*	
109_7	002050N_81N_96N	002057N_87N_111N	14.4	*	
109_8	002050N_81N_96N	002065N_106N_107N	12.2	*	
109_9	002050N_81N_96N	002043N_61N_110N	10.8	*	
109_10	002050N_81N_96N	002N_88N_114N_118N	5.82	*	
110_1	002036N_39N_93N	002N_41N_79N_100N	10.3	*	
110_2	002036N_39N_93N	002037N_42N_70N	9.72	*	
110_3	002036N_39N_93N	002N_41N_79N_100N	8.06	*	
110_4	002036N_39N_93N	002038N_67N_94N	9.73	*	
110_5	002036N_39N_93N	002073N_84N_89N	12.4	*	
110_6	002036N_39N_93N	002N_88N_114N_118N	3.41	*	
110_7	002036N_39N_93N	002056N_86N_123N	11.8	*	
110_8	002036N_39N_93N	002N_41N_79N_100N	12.3	*	
110_9	002036N_39N_93N	002064N_66N_74N_112N	8.13	*	
110_10	002036N_39N_93N	002054N_77N_82N	9.14	*	
135_1	002045N_109N_117N	002072N_91N_95N	1.3	*	
135_2	002045N_109N_117N	002056N_86N_123N	10.5	*	
135_3	002045N_109N_117N	002045N_109N_117N	1.59	*	**probable self**
135_4	002045N_109N_117N	002057N_87N_111N	12.8	*	
135_5	002045N_109N_117N	002050N_81N_96N	7.34	*	
135_6	002045N_109N_117N	002038N_67N_94N	6.28	*	
135_7	002045N_109N_117N	002036N_39N_93N	11.9	*	
135_8	002045N_109N_117N	002055N_80N_103N	3.59	*	
135_9	002045N_109N_117N	002036N_39N_93N	8.07	*	
135_10	002045N_109N_117N	002040N_63N_105N	11.2	*	

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Progeny ID	"Known" female parent	Most Likely male parent	LOD	Confidence	
150_1	002052N_85N_92N	002050N_81N_96N	11.2	*	
150_2	002052N_85N_92N	002047N_48N_51N	2.09	*	
150_3	002052N_85N_92N	002065N_106N_107N	8.84	*	
150_4	002052N_85N_92N	002052N_85N_92N	8.69	*	**probable self**
150_5	002052N_85N_92N	002072N_91N_95N	0.101	*	
150_6	002052N_85N_92N	002055N_80N_103N	13.1	*	
150_7	002052N_85N_92N	002058N_76N_121N	10.8	*	
150_8	002052N_85N_92N	002052N_85N_92N	14.4	*	**probable self**
150_9	002052N_85N_92N	002059N_75N_108N	11.5	*	
150_10	002052N_85N_92N	002071N	9.14	*	
153_1	002072N_91N_95N	002072N_91N_95N	11	*	**probable self**
153_2	002072N_91N_95N	002038N_67N_94N	9.56	*	
153_3	002072N_91N_95N	002038N_67N_94N	8.01	*	
153_4	002072N_91N_95N	002065N_106N_107N	12.7	*	
153_5	002072N_91N_95N	002062N_68N_113N	1.42	*	
153_6	002072N_91N_95N	002072N_91N_95N	12.4	*	**probable self**
153_7	002072N_91N_95N	002072N_91N_95N	12	*	**probable self**
153_8	002072N_91N_95N	002064N_66N_74N_112N	9.29	*	
153_9	002072N_91N_95N	002072N_91N_95N	12.6	*	**probable self**
153_10	002072N_91N_95N	002057N_87N_111N	12.4	*	
158_1	002N_83N_90N_104N	002073N_84N_89N	4.33	*	
158_2	002N_83N_90N_104N	002073N_84N_89N	7.59	*	
158_3	002N_83N_90N_104N	002073N_84N_89N	7.53	*	
158_4	002N_83N_90N_104N	002073N_84N_89N	9.98	*	
158_5	002N_83N_90N_104N	002116N_122N	14.6	*	
158_6	002N_83N_90N_104N	002073N_84N_89N	9.47	*	
158_7	002N_83N_90N_104N	002073N_84N_89N	9.83	*	
158_8	002N_83N_90N_104N	002073N_84N_89N	10.3	*	
158_9	002N_83N_90N_104N	002073N_84N_89N	9.78	*	
158_10	002N_83N_90N_104N	002073N_84N_89N	8.75	*	
163_1	002060N_115N_119N	002073N_84N_89N	8.15	*	
163_2	002060N_115N_119N	002073N_84N_89N	7.56	*	
163_3	002060N_115N_119N	002073N_84N_89N	9.64	*	
163_4	002060N_115N_119N	002073N_84N_89N	9.15	*	
163_5	002060N_115N_119N	002057N_87N_111N	11	*	
163_6	002060N_115N_119N	002069N_98N_99N	9.09	*	
163_7	002060N_115N_119N	002073N_84N_89N	6.99	*	
163_8	002060N_115N_119N	002055N_80N_103N	10.8	*	
163_9	002060N_115N_119N	002072N_91N_95N	11.5	*	
163_10	002060N_115N_119N	002073N_84N_89N	7.85	*	
164_1	002116N_122N	002N_88N_114N_118N	4.71	*	
164_2	002116N_122N	002062N_68N_113N	14.2	*	
164_3	002116N_122N	002058N_76N_121N	8.87	*	

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Progeny ID	"Known" female parent	Most Likely male parent	LOD	Confidence
164_4	002116N_122N	002N_41N_79N_100N	1.4	*
164_5	002116N_122N	002052N_85N_92N	10.5	*
164_6	002116N_122N	002056N_86N_123N	3.59	*
164_7	002116N_122N	002043N_61N_110N	4.51	*
164_8	002116N_122N	002072N_91N_95N	1.03	*
164_9	002116N_122N	002056N_86N_123N	7.64	*
164_10	002116N_122N	002N_41N_79N_100N	3.5	*
423_1	002065N_106N_107N	002040N_63N_105N	14.2	*
423_2	002065N_106N_107N	002055N_80N_103N	13.5	*
423_3	002065N_106N_107N	002073N_84N_89N	7.37	*
423_4	002065N_106N_107N	002073N_84N_89N	10.6	*
423_5	002065N_106N_107N	002073N_84N_89N	6.71	*
423_6	002065N_106N_107N	002055N_80N_103N	9.07	*
423_7	002065N_106N_107N	002043N_61N_110N	5.85	*
423_8	002065N_106N_107N	002073N_84N_89N	6.91	*
423_9	002065N_106N_107N	002073N_84N_89N	9.93	*
423_10	002065N_106N_107N	002037N_42N_70N	7.54	*

Appendix Table 2. Publicly available microsatellite markers used for the parental reconstruction of open-pollinated *E.nitens* progenies.

Primer Name	Actual Size (Pig Tails)	Dye	PIC	Forward	Reverse	Observed heterozygosity	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	175-192	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
FRMSA2	109-121	PET	0.109	CGTCGTACTCTAGTCAATGC	ATCCTCCGCTTAAGAGGCTC	0.088	3

Average = 9.2