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## EUCALYPT COOPERATIVE

Report No. 7

# Determining the effective pollination among clones of *Eucalyptus fastigata* using SSR markers.

by
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Date: August 2007

SCION
Next generation biomaterials

Eucalypt Cooperative
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THE JOINT FORCES OF CSIRO & SCION

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# DETERMINING THE EFFECTIVE POLLINATION AMONG CLONES OF *EUCALYPTUS FASTIGATA* USING SSR MARKERS

By

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## SUMMARY

Parentage reconstruction using 18 selected microsatellite (SSR) markers was carried out on open-pollinated (OP) seedling families of *E. fastigata,* following the successful reconstruction of parentage of OP seedlings of *Eucalyptus nitens* (Gea *et al*, 2006). Seed was collected from the mid crown of 10 clones in a 37-clone clonal archive. Genomic DNA was extracted from foliage collected from ten seedlings of the ten OP families.

The number of seedlings with both parents identified was 90 of the 99 tested. Seven progeny had paternal parents that were not consistent with orchard trees, and are presumably products of contaminating pollen from outside the orchard. Two other progeny were not consistent with their expected mothers. The selfing rate was 8.9%.

## INTRODUCTION

*Eucalyptus fastigata* is at present being grown on a limited scale in New Zealand for the production of pulpwood, and older plantations have been successfully utilised for sawn timber, especially flooring. The genetic improvement programme started in 1980 with the planting of 126 open-pollinated progenies. The parents were mainly of various provenances from the natural range in eastern Victoria and New South Wales (Cannon & Shelbourne 1991, 1993), some local New Zealand landrace selections and a few South African families. In 1995 this breeding population was expanded by

180 open-pollinated families from parents chosen in native populations and in plantations in South Africa. Forwards selections from the original 1980 progeny/provenance trials have not yet been progeny tested because of lack of seed on the parent ortets. These plus-tree selections have instead been grafted and established as a clonal archive. For reasons of economy and operational efficiency for improvement of what is only a minor species, the breeding strategy utilises open- as opposed to control-pollinated progeny.

Gea *et al* (2007), and subsequently McConnochie *et al*. (in prep.), successfully developed for *E. nitens* the use of 15 microsatellite markers (SSR) to reveal the parental identity of seedlings of open-pollinated families collected within a clonal seed orchard. They carried out reconstruction of male and female parentage on 10 seedlings per clonal seed parent family of a sample of 10 clones from the 30-clone orchard. The orchard offspring from seed collected in both the lower- and mid-zone of the crown showed high levels of outcrossing and the selfing rate among the orchard clones was 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.

# METHODS AND MATERIALS

In 1998, a clonal archive of *E.fastigata* was established on an ex-pasture site in Te Waerenga forest near Rotorua which is planted predominantly in *E.nitens* for pulpwood production. The clones in the archive had been forwards-selected at age 17 years from two sites in the central North Island of the 1980-planted open-pollinated progeny test of 126 seedlots, (Table 1). They were selected for diameter growth, stem form, branching habit, basic density and absence of internal checking. 48 grafts are established in the clonal archive. Most clones are represented by one ramet in the archive, seven clones have two ramets, and two clones have three ramets randomly positioned across the site. Three families are represented by two or more clones.

A set of 18 markers was developed for *E.fastigata,* (Table 2), and nine are in common with the 15 marker set used for *E.nitens*. The markers were tested across all trees in the Te Waerenga archive and an additional 30 unrelated individuals were genotyped to provide a better estimate of allele frequencies. Genomic DNA was extracted from leaf material from each ramet in the archive and used to obtain a consistent multi-locus genotype of each of the clones.

The technique employed for this study is a reliable and widely-used genomic DNA isolation method similar to that described by Stacey & Isaac (1994). OP seed was then collected from a sample 10 clones and seedlings raised in containers. Leaf material of 10 randomly selected seedlings per OP family was taken and the 18 SSR markers used to identify the parentage.

### *Cervus analyses*

Parentage analyses were carried out using Cervus 2.0 (Marshall *et al*. 1998), following the same process as in Gea *et al*. (2007). 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 output gives a list of possible pollen parents of each individual progeny seedling, along with their LOD scores, Delta values and a confidence level. 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 AND DISCUSSION

### *Comparison of candidate parent genotypes*

Multilocus genotypes for all 48 grafted trees and thus potential parents in the clonal archive were compared for consistency with their 'known" identity. Trees with identical genotype codes have the same multilocus genotype and are considered to be ramets of a clone. Some grafts were known to be of the same clone, while a few others have different multilocus genotypes than expected. For example, SampleID 002300N (TagID 1264_16) had an identical genotype to SampleID 002307N (TagID 1264_24) at all 18 SSR marker loci. These trees are highly likely to be ramets of the same clone, mis-identification having occurred during propagation or planting of the archive.

The identity of the 48 trees in the archive was accordingly revised to include 37 clones (Table 3). These 37 different genotypes were then used as the set of candidate paternal parent genotypes in the paternity studies.

## *Consistency of progeny genotypes with expected maternal genotypes*

Multilocus genotype arrays were used to test each seedling of each family against the 37 clones in the clonal archive, with no prior expectations of parentage given to Cervus. All expected 10 clonal seed parents appeared near the top of the list of possible parents, with relatively 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. Two progeny were not consistent with their expected maternal genotype.

## *Paternal genotype identification*

The 97 seedling genotypes (out of 99) that were consistent with being from the expected clonal seed parents were reanalysed in Cervus, assuming "known" maternal genotypes, to determine the most likely paternal genotype. From this analysis, self-pollinated offspring were identified if the maternal genotype was also the most likely paternal genotype; stray or "polluting" pollen from outside the set of candidate parents was detected if the most likely paternal parent has a LOD score less than zero; and in all other cases a most-likely candidate parent was identified from within the clonal archive (Appendix Table 1).

The number of seedlings with both parents identified was 90 of the 99 tested (Table 4). Seven seedlings had a paternal parent that was not consistent with any of the orchard trees, suggesting possible pollen flow from outside of the orchard, and eight progeny were apparent selfs. The selfing rate ranged from zero (for 6 of 10 clones) to 30% for two clones and averaged 8.9%. Two progeny, 78_10 and 87_9, were not consistent with their genotype of their expected seed parent or with the other nine offspring assayed from the same clone. Individual clones from the same family were able to be distinctly identified as a paternal parent.

The number of clones represented as pollen parents across all samples with full parental reconstruction was 23 out of 24 clones producing flowers in the

archive. Five clones contributed as pollen parents to 55% of the seedlings (Clone nos. 78,27,35,65,68). 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 9 seedlings of Clone 24, that were successfully matched to both a mother and father, there were eight 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 specific pollens.

## APPLICATIONS IN TREE BREEDING

It has now been shown that the parentage of open-pollinated progenies from clonal orchards or archives of *Eucalyptus nitens* and *E. fastigata* can be successfully identified using microsatellite marker sets dedicated to each species. The implications for modifying the breeding strategy and improving the efficiency of the breeding cycle are evident. In future, provided that forward selections are grafted and established in clonal archives or seed orchards and genotyped, mating these selections to form the next generation of the breeding population can be effected by allowing insect open-pollination. Parental identity of forward selections can be achieved and the number of related parents in the breeding population can be managed to reduce inbreeding.

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**Table 1.** *E. fastigata* Clones in Te Waerenga Clonal Archive .

| Clone No. | Family Code | Origin | No. Ramets | Clone No. | Family Code | Origin | No. Ramets |
|---|---|---|---|---|---|---|---|
| 891-3 | 41 | Robertson | 1 | 55 | 122 | Stewarts Brook | 2 |
| 6 | 2 | Oberon | 1 | 61 | 148 | Cambridge, NZ | 1 |
| 16 | 43 | Robertson | 1 | 63 | 7 | Oberon | 2 |
| 18 | 30 | Bombala | 2 | 64 | 72 | Tallaganda | 1 |
| 22 | 117 | Stewarts Brook | 3 | 65 | 109 | Lake Mangamahoe, NZ | 1 |
| 23 | 64 | Yetholme | 1 | 68 | 19 | Rossi | 1 |
| 24 | 39 | Robertson | 1 | 71 | 103 | Oakura, NZ | 1 |
| 27 | 125 | Natal | 1 | 72 | 69 | Tallaganda | 1 |
| 32 | 11 | Oberon | 2 | 75 | 84 | FRI, NZ | 1 |
| 33 | 123 | Ngahinapouri, NZ | 1 | 76 | 5 | Oberon | 1 |
| 35 | 125 | Natal | 2 | 78 | 24 | Rossi | 3 |
| 37 | 15 | Rossi | 1 | 79 | 105 | Oakura, NZ | 1 |
| 39 | 2 | Oberon | 2 | 83 | 58 | Oberon | 1 |
| 42 | 100 | Oakura, NZ | 1 | 84 | 5 | Oberon | 1 |
| 43 | 8 | Oberon | 1 | 87 | 16 | Rossi | 1 |
| 44 | 99 | Oakura, NZ | 1 | 90 | 92 | Tikitere, NZ | 1 |
| 45 | 94 | Waimana, NZ | 1 | 93 | 45 | Robertson | 1 |
| 48 | 22 | Rossi | 1 | 94 | 104 | Oakura, NZ | 1 |
| 50 | 125 | Natal | 2 | | | | |

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

| Primer Name | Actual Size (Pig Tails) | Dye | PIC | Forward | Reverse | Observed heterozygosity | No. alleles |
|---|---|---|---|---|---|---|---|
| Es076 | 118-176 | PET | 0.920 | AATGCTGCTGTAGACGATGC | GTTTCTTAAGACAAATCAAGCAAGTCAGC | 0.836 | 28 |
| Es054 | 95-117 | FAM | 0.686 | GGAAGAAATCAAACTGGACACC | GTTTCTTTTTGCGACTACCATTTTCACC | 0.632 | 11 |
| Es140 | 128-175 | NED | 0.868 | GCTCATTGTACTGCACAGAGG | GTTTCTTAAGGCACCAACAGTACCTGG | 0.826 | 25 |
| Es211 | 100-118 | VIC | 0.811 | GGGAGAGCTGATTGAGTAATTG | GTTTCTTGCTGAGAATGGAAGCACATC | 0.768 | 12 |
| FRMSA1 | 208-237 | NED | 0.855 | GCCTTGAAAGAGAGGGAGAG | GTTTCTTCCACACGGAAGAGCTTCAGC | 0.712 | 15 |
| FRMSA2 | 116-124 | PET | 0.109 | CGTCGTACTCTAGTCAATGC | ATCCTCCGCTTAAGAGGCTC | 0.088 | 3 |
| FRMSA4 | 300-325 | VIC | 0.145 | GACGATGAAGATGAGGATGG | GTTTCTTGCAACAGCGAAACTGAAAAT | 0.087 | 5 |
| En16 | 155-199 | PET | 0.924 | TTTCCTCTTCACGCACTCG | GTTTCTTCTCCCGGGCCCTGTA | 0.940 | 22 |
| EMBRA7 | 129-143 | VIC | 0.334 | CACACCGTGTCAGTTAGC | GTTTCTTAATAAGGAGGATTCCATGG | 0.313 | 5 |
| EMBRA64 | 253-265 | PET | 0.723 | CAGAACCCAGCGGAGGA | GTTTCTTAGCTCCCTTCACAAGGTA | 0.739 | 9 |
| EMBRA63 | 184-230 | PET | 0.834 | CATCTGGAGATCGAGGAA | GTTTCTTGAGAGAAGGATCATGCCA | 0.725 | 19 |
| EMBRA44 | 195-229 | NED | 0.892 | GGGGTTTGTTCTGCTTAG | GTTTCTTCCAAAGAGTTCAGCTGTG | 0.721 | 15 |
| EMBRA39 | 129-153 | PET | 0.845 | GCATTCGTACTCATTTTCAA | GTTTCTTGCATCGAGAGTGGATTAGTT | 0.585 | 12 |
| EMBRA29 | 242-292 | FAM | 0.877 | CTTCGCTCACATCAGTCTC | GTTTCTTCAATCGAGTCAATAACATTCA | 0.600 | 20 |
| EMBRA12 | 115-127 | FAM | 0.632 | AGGATTTGTGGGGCAAGT | GTTTCTTGTTCCCCATTTTCATGTCC | 0.652 | 6 |
| EMBRA11 | 116-168 | PET | 0.898 | GCTTAGAATTTGCCTAAACC | GTTTCTTGTAAAATCCATGGGCAAG | 0.768 | 20 |
| Eg99 | 180-201 | NED | 0.717 | CTCATCAGCCTCCGAAACAC | GTTTCTTGAAAGGAGGGACCTTTGAGG | 0.667 | 8 |
| Eg126 | 342-375 | NED | 0.852 | GAGGTCGAACGCAAGATAGC | GTTTCTTTCTTATGGGGACATCAAGCC | 0.855 | 13 |
| | | | | | | | Average = 13.8 |

**Table 3.** Identity of ramets as candidate parent genotypes

| Clone _Ramet code | Type | Genotype code | Clone _Ramet code | Type | Genotype code |
|---|---|---|---|---|---|
| 3 | Putative paternal | 1 | 50_1 | Putative paternal | 16 |
| 6 | Putative paternal | 2 | 50_2 | Putative paternal | 16 |
| 16 | Putative paternal | 3 | 55_1 | Putative paternal | 20 |
| 18_1 | Putative paternal | 4 | 55_2 | Putative paternal | 20 |
| 18_2 | Putative paternal | 4 | 61 | Maternal | 21 |
| 22_1 | Maternal | 5 | 63_1 | Putative paternal | 22 |
| 22_2 | Maternal | 6 | 63_2 | Putative paternal | 22 |
| 22_3 | Maternal | 7 | 64 | Putative paternal | 23 |
| 23 | Maternal | 8 | 65 | Putative paternal | 24 |
| 24 | Maternal | 3 | 68 | Putative paternal | 25 |
| 27 | Maternal | 9 | 71 | Putative paternal | 26 |
| 32_1 | Putative paternal | 10 | 72 | Maternal | 27 |
| 32_2 | Putative paternal | 10 | 75 | Putative paternal | 28 |
| 33 | Putative paternal | 11 | 76 | Putative paternal | 29 |
| 35_1 | Putative paternal | 12 | 78_1 | Maternal | 30 |
| 35_2 | Putative paternal | 12 | 78_2 | Maternal | 30 |
| 37 | Putative paternal | 13 | 78_3 | Maternal | 30 |
| 39_1 | Maternal | 14 | 79 | Putative paternal | 31 |
| 39_2 | Maternal | 14 | 83 | Putative paternal | 32 |
| 42 | Putative paternal | 15 | 84 | Putative paternal | 33 |
| 43 | Putative paternal | 16 | 87 | Maternal | 34 |
| 44 | Maternal | 17 | 90 | Maternal | 35 |
| 45 | Maternal | 18 | 93 | Putative paternal | 36 |
| 48 | Putative paternal | 19 | 94 | Putative paternal | 37 |

**Table 4.** Number of fathers and selfs per clone among the open-pollinated seedlings with full parental reconstruction

| Clone No. | 23 | 24 | 27 | 39 | 44 | 61 | 72 | 78 | 87 | 90 |
|---|---|---|---|---|---|---|---|---|---|---|
| No. seedlings with full parental reconstruction | 9 | 9 | 8 | 8 | 10 | 10 | 10 | 8 | 9 | 9 |
| No. fathers | 5 | 8 | 7 | 6 | 6 | 5 | 4 | 4 | 5 | 6 |
| No. selfs | 3 | 0 | 1 | 0 | 0 | 0 | 0 | 3 | 1 | 0 |
| Origin | Aust | Aust | S.A | Aust | NZ | NZ | Aust | Aust | Aust | NZ |

**Appendix Table 1.** *E. fastigata* paternity results

| Progeny ID | "Known" female parent | Most likely male parent | LOD | Confidence | |
|---|---|---|---|---|---|
| 23_1 | 1264_23 | 1264_23 | 14.070 | * | probable self |
| 23_2 | 1264_23 | 1264_35 | 7.978 | * | |
| 23_3 | 1264_23 | 1264_42 | 10.267 | * | |
| 23_4 | 1264_23 | 1264_23 | 10.559 | * | probable self |
| 23_5 | 1264_23 | 1264_27 | 5.764 | * | |
| 23_6 | 1264_23 | | | | no male parent identified in orchard |
| 23_7 | 1264_23 | 1264_45 | 13.305 | * | |
| 23_8 | 1264_23 | 1264_45 | 13.792 | * | |
| 23_9 | 1264_23 | 1264_23 | 12.625 | * | probable self |
| 23_10 | 1264_23 | 1264_35 | 8.372 | * | |
| | | | | | |
| 24_1 | 1264_16_24 | 1264_93 | 5.388 | * | |
| 24_2 | 1264_16_24 | 1264_63 | 15.005 | * | |
| 24_3 | 1264_16_24 | 1264_45 | 17.330 | * | |
| 24_4 | 1264_16_24 | 1264_42 | 6.786 | * | |
| 24_5 | 1264_16_24 | 1264_93 | 5.510 | * | |
| 24_6 | 1264_16_24 | 1264_55 | 0.862 | * | |
| 24_7 | 1264_16_24 | | | | no male parent identified in orchard |
| 24_8 | 1264_16_24 | 1264_90 | 4.848 | * | |
| 24_9 | 1264_16_24 | 1264_22_3 | 0.646 | * | |
| 24_10 | 1264_16_24 | 1264_27 | 6.805 | * | |
| | | | | | |
| 27_1 | 1264_27 | 1264_65 | 11.026 | * | |
| 27_2 | 1264_27 | 1264_35 | 11.366 | * | |
| 27_3 | 1264_27 | 1264_27 | 6.117 | * | probable self |
| 27_4 | 1264_27 | | | | no male parent identified in orchard |
| 27_6 | 1264_27 | 1264_16_50 | 0.169 | * | |
| 27_7 | 1264_27 | 1264_35 | 6.971 | * | |
| 27_8 | 1264_27 | 1264_42 | 12.387 | * | |
| 27_9 | 1264_27 | 1264_44 | 9.453 | * | |
| 27_10 | 1264_27 | 1264_33 | 12.980 | * | |
| | | | | | |
| 39_1 | 1264_39 | 1264_35 | 17.948 | * | |
| 39_2 | 1264_39 | 1264_44 | 8.329 | * | |
| 39_3 | 1264_39 | 1264_76 | 2.763 | * | |
| 39_4 | 1264_39 | 1264_45 | 18.643 | * | |
| 39_5 | 1264_39 | | | | no male parent identified in orchard |
| 39_6 | 1264_39 | 1264_84 | 5.658 | * | |
| 39_7 | 1264_39 | 1264_35 | 13.882 | * | |
| 39_8 | 1264_39 | 1264_65 | 11.714 | * | |
| 39_9 | 1264_39 | 1264_35 | 8.317 | * | |

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| Progeny ID | "Known" female parent | Most likely male parent | LOD | | Confidence |
|---|---|---|---|---|---|
| 39_10 | 1264_39 | | | | no male parent identified in orchard |
| | | | | | |
| 44_1 | 1264_44 | 1264_22_3 | 1.019 | * | |
| 44_2 | 1264_44 | 1264_65 | 6.933 | * | |
| 44_3 | 1264_44 | 1264_65 | 5.877 | * | |
| 44_4 | 1264_44 | 1264_35 | 9.748 | * | |
| 44_5 | 1264_44 | 1264_65 | 16.356 | * | |
| 44_6 | 1264_44 | 1264_39 | 10.376 | * | |
| 44_7 | 1264_44 | 1264_27 | 13.056 | * | |
| 44_8 | 1264_44 | 1264_23 | 3.765 | * | |
| 44_9 | 1264_44 | 1264_27 | 7.986 | * | |
| 44_10 | 1264_44 | 1264_35 | 10.452 | * | |
| | | | | | |
| 61_1 | 1264_61 | 1264_78 | 11.557 | * | |
| 61_2 | 1264_61 | 1264_78 | 7.627 | * | |
| 61_3 | 1264_61 | 1264_16_50 | 4.510 | * | |
| 61_4 | 1264_61 | 1264_78 | 13.419 | * | |
| 61_5 | 1264_61 | 1264_68 | 7.220 | * | |
| 61_6 | 1264_61 | 1264_39 | 4.150 | * | |
| 61_7 | 1264_61 | 1264_68 | 8.718 | * | |
| 61_8 | 1264_61 | 1264_68 | 7.142 | * | |
| 61_9 | 1264_61 | 1264_78 | 15.006 | * | |
| 61_10 | 1264_61 | 1264_65 | 7.342 | * | |
| | | | | | |
| 72_1 | 1264_72 | 1264_78 | 12.250 | * | |
| 72_2 | 1264_72 | 1264_78 | 8.350 | * | |
| 72_3 | 1264_72 | 1264_78 | 17.699 | * | |
| 72_4 | 1264_72 | 1264_64 | 12.707 | * | |
| 72_5 | 1264_72 | 1264_65 | 0.234 | * | |
| 72_6 | 1264_72 | 1264_78 | 11.702 | * | |
| 72_7 | 1264_72 | 1264_68 | 8.353 | * | |
| 72_8 | 1264_72 | 1264_64 | 10.682 | * | |
| 72_9 | 1264_72 | 1264_68 | 3.296 | * | |
| 72_10 | 1264_72 | 1264_65 | 12.379 | * | |
| | | | | | |
| 78_1 | 1264_78 | 1264_68 | 10.230 | * | |
| 78_2 | 1264_78 | 1264_22_1 | 1.521 | * | |
| 78_3 | 1264_78 | 1264_72 | 11.729 | * | |
| 78_4 | 1264_78 | 1264_78 | 13.511 | * | probable self |
| 78_5 | 1264_78 | 1264_68 | 8.707 | * | |
| 78_6 | 1264_78 | 1264_68 | 8.323 | * | |
| 78_7 | 1264_78 | 1264_78 | 14.970 | * | probable self |
| 78_8 | 1264_78 | | | | no male parent identified in orchard |

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| Progeny ID | "Known" female parent | Most likely male parent | LOD | Confidence | |
|---|---|---|---|---|---|
| 78_9 | 1264_78 | 1264_78 | 13.581 | * | probable self |
| 78_10 | | | | | does not match expected mother |
| | | | | | |
| 87_1 | 1264_87 | 1264_68 | 1.141 | * | |
| 87_2 | 1264_87 | 1264_78 | 9.955 | * | |
| 87_3 | 1264_87 | 1264_64 | 11.451 | * | |
| 87_4 | 1264_87 | 1264_16_50 | 6.142 | * | |
| 87_5 | 1264_87 | 1264_78 | 9.319 | * | |
| 87_6 | 1264_87 | 1264_87 | 0.567 | * | probable self |
| 87_7 | 1264_87 | 1264_78 | 15.639 | * | |
| 87_8 | 1264_87 | 1264_78 | 5.721 | * | |
| 87_9 | | | | | does not match expected mother |
| 87_10 | 1264_87 | 1264_68 | 4.861 | * | |
| | | | | | |
| 90_1 | 1264_90 | | | | no male parent identified in orchard |
| 90_2 | 1264_90 | 1264_33 | 12.973 | * | |
| 90_3 | 1264_90 | 1264_16_24 | 10.616 | * | |
| 90_4 | 1264_90 | 1264_35 | 18.260 | * | |
| 90_5 | 1264_90 | 1264_42 | 7.533 | * | |
| 90_6 | 1264_90 | 1264_37 | 0.438 | * | |
| 90_7 | 1264_90 | 1264_33 | 15.986 | * | |
| 90_8 | 1264_90 | 1264_27 | 4.581 | * | |
| 90_9 | 1264_90 | 1264_27 | 7.047 | * | |
| 90_10 | 1264_90 | 1264_42 | 10.777 | * | |