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Construction of Microsatellite Linkage Maps for *Corymbia*

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Abstract

The genus *Corymbia* is closely related to the genus *Eucalyptus*, and like *Eucalyptus* contains tree species that are important for sub-tropical forestry. *Corymbia's* close relationship with *Eucalyptus* suggests genetic studies in *Corymbia* should benefit from transfer of genetic information from its more intensively studied relatives. Here we report a genetic map for *Corymbia* spp. based on microsatellite markers identified *de novo* in *Corymbia* sp or transferred from *Eucalyptus*. A framework consensus map was generated from an outbred F_2 population (n = 90) created by crossing two unrelated *Corymbia torelliana* x *C. citriodora* subsp. *variegata* F_1 trees. The map had a total length of 367 cM (Kosambi) and was composed of 46 microsatellite markers distributed across 13 linkage groups (LOD 3). A high proportion of *Eucalyptus* microsatellites (90%) transferred to *Corymbia*. Comparative analysis between the *Corymbia* map and a published *Eucalyptus* map identified eight homeologous linkage groups in *Corymbia* with 13 markers mapping on one or both maps. Further comparative analysis was limited by low power to detect linkage due to low genome coverage in *Corymbia*, however, there was no convincing evidence for chromosomal structural differences because instances of non-synteny were associated with large distances on the *Eucalyptus* map. Segregation distortion was primarily restricted to a single linkage group and due to a deficit of hybrid genotypes, suggesting that hybrid inviability was one factor shaping the genetic composition of the F_2 population in this inter-subgeneric hybrid. The conservation of microsatellite loci and synteny between *Corymbia* and *Eucalyptus* suggests there will be substantial value in exchanging information between the two groups.

Key words: Corymbia torelliana; *Corymbia citriodora* subsp. *variegata*; genetic map; hybrid inviability; genetic marker; tree improvement; marker-aided selection.

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Introduction

The development of genetic maps and a comparative analysis of maps amongst related plant groups has provided new insights into genome structure, organisation and evolution, with implications for plant improvement, gene pool conservation and management (NEWBURY and PATERSON, 2003). Comparative studies are particularly valuable for commercial taxa which are resource poor in genetic information (eg. DNA markers or DNA sequence data) but where they have near relatives that are resource rich (BROWN *et al.,* 2001). This situation exists for the genera *Eucalyptus* and *Corymbia*; most eucalypts of commercial importance belong to the genus *Eucalyptus*, the great majority of gene sequence and marker information is derived from a few *Eucalyptus* species, *E. grandis*, *E. urophylla* and *E. globulus.* The genus *Corymbia* has 113 species in 7 sections and includes

important commercial taxa for forestry in the Section Politaria (i.e. spotted gums) and Section Cadagaria (*Corymbia torelliana*) (HILL and JOHNSON, 1995; LEE, 2005), but for which there is comparatively little genetic information. A key question, therefore, is how relevant and to what extent genetic information can be transferred between the two taxa.

Comparative map analysis has revealed that high degrees of synteny (genetic loci are located upon the same chromosome) and colinearity (congruent ordering of loci on chromosomes) may exist for quite phylogenetically divergent groups in both plants and animals (BONIERBALE *et al.,* 1988; LAGERCRANTZ and LYDIATE, 1996; MOORE *et al.,* 1995; O'BRIEN *et al.,* 1999). Amongst tree species, extensive synteny is evident in species of the genus *Pinus*, with little evidence for gross structural rearrangement amongst sub-sections of hard pines (Genus *Pinus* Subgenus *Pinus*) (BROWN *et al.,* 2001; DEVEY *et al.,* 1999), including taxa that may have diverged as long as 70 MYA (KOMULAINEN, 2003). Conservation of genome structure and organisation is also expected to be high amongst *Eucalyptus* sp. which should allow efficient exchange of genetic information within the genus (BRONDANI *et al.,* 2002; MARQUES *et al.,* 2002). Several recent comparative studies are supportive of no significant evidence of rearrangement amongst genetic maps of representatives from three sections of the subgenus *Symphyomyrtus*, section *Latoangulatae* (*E. grandis*), Maidenaria (*E. globulus*) and *Exsertaria* (*E. tereticornis*) (FREEMAN, 2006; MYBURG *et al.,* 2003).

Eucalypts in the broad sense encompass three genera (*Angophora*, *Corymbia* and *Eucalyptus*). *Corymbia* (the bloodwoods and ghost gums) were initially classified as a subgenus within *Eucalyptus* but were given formal genus status in 1995 (HILL and JOHNSON, 1995). Although initially controversial, this reclassification has become widely accepted over the past decade as it conforms with phylogenetic relationships which show a basal dichotomy in the eucalypts with an Angophoroid clade (*Angophora* and *Corymbia*) and a non-Angophoroid clade (*Eucalyptus* s.s.) (HILL and JOHNSON, 1995; LADIGES *et al.,* 1995; STEANE *et al.,* 2002; UDOVICIC *et al.,* 1995). Fossil evidence suggests these major evolutionary lineages had diverged by the Middle Miocene (14–17 MYA) (HOLMES *et al.,* 1983).

Physical genome characteristics of the two groups suggest there may be substantive differences in the organisation of the two genomes. The evidence available for DNA content indicates that *Corymbia* sp have around $^1/_2$ the DNA content of a typical Symphyomyrt (i.e. *E. globulus*; *E. grandis*) (GRATTAPAGLIA and BRADSHAW, 1994). Differences in the karyotypes for the two groups, however, are subtle. All eucalypts have a haplotype chromosome number of 11 and extremely small uniform chromosomes (HAQUE, 1984). Detailed comparison of karyotype morphology has indicated that it is possible to distinguish *E. maculata* (now *Corymbia maculata*) from other *Eucalyptus* spp because of its more symmetrical karyotype (MATSUMOTO *et al.,* 2000). The degree to which the difference in physical genome size impacts genome structure and organisation is unknown. For example, is the difference in genome size of *Corymbia* the result of a few major gross structural rearrangements (i.e. deletions) or are the changes more subtle, a consequence of smaller deletions dispersed throughout the genome? Recently, evidence for this second hypothesis was reported to explain differences in genome sizes between *E. grandis* and *E. globulus* (MYBURG et al., 2003).

This paper reports the first genetic map for *Corymbia* and a comparative analysis with published *Eucalyptus grandis* map. We report a consensus map based on two *Corymbia torelliana* x *C. citriodora* subsp. *variegata* F_1 hybrid individuals. An outbred F_2 population was selected for study because the F_1 hybrid is of emerging commercial importance for tropical hardwood plantations in Australia, and the F_2 is expected to segregate widely making it ideal for QTL detection experiments. The map was constructed from microsatellite markers developed de novo in *Corymbia citriodora* subsp. *variegata* (henceforth subsp. *variegata*) and by transference of markers identified in *Eucalyptus* spp.

Methods

Mapping population

The mapping population was an outbred F_2 intersectional hybrid family. First, two unrelated F_1 families were generated by hand pollinating two individuals of *Corymbia torelliana* (Section Cadagaria) individuals with pollen from two individuals of subsp. *variegata* (Section Politaria). An F_2 was generated by intermating a select F_1 from each family (*Figure 1*). Four hundred seed were sown in Queensland Department of Primary Industries and Fisheries, Gympie Glasshouse on the 12th November 2004. Seed germination and early seedling survival was low with only 208 seedlings surviving on 31st Jan 2005 and 154 surviving at 6 mths post-sowing. In January 2005, foliage was obtained from the 208 surviving seedlings, the two F_1 parent trees and the four grandparents, and stored frozen at –20 °C until required for DNA extraction. A set of 90 F_2, the two parents and four grandparents were used to study marker inheritance and segregation.

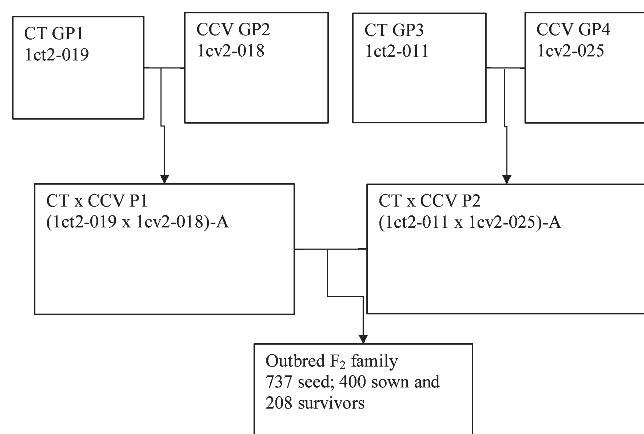

Figure 1. – Pedigree of *C. torelliana* x *C. citriodora* subsp. *variegata* outbred F_2 mapping population. 1ct2-019 and 1ct2-011 were the *C. torelliana* maternal grandparents and 1cv2-018 and 1cv2025 the paternal *C. citriodora* subsp. *variegata* grandparents. (1ct2-011 x 1cv2-025)-A and (1ct2-019 x 1cv2-018)-A were the F_1 parents crossed to form the F_2 family.

DNA extraction

DNA was extracted according to the frozen tissue protocol with the DNeasy 96 Plant kit (Qiagen GmbH, Hilden, Germany) with the following modifications. The amount of tissue was reduced to 25–40 mg, PVP (2%) was added to Buffer AP1 and the volume increased to 800 ul per sample. DNA was eluted into a final volume of 2 x 100 ul aliquots in Buffer AE.

Microsatellite markers isolated from subsp. variegata

Microsatellite loci were identified *de novo* in subsp. *variegata*. Primer sequences and methods used to isolate 14 of these loci have been previously described (JONES *et al.,* 2001). A further 14 primer-pairs were developed for this study (*Table 1*). Suitability of loci for mapping and population studies was evaluated by amplifying each locus on a test panel of 21 individuals including the mapping pedigree and a set of 12 diverse subsp. *variegata* individuals. Polymorphism information content (PIC) for each marker was calculated using GenAlEx software (PEAKALL and SMOUSE, 2001).

Microsatellite markers transferred from Eucalyptus spp.

A set of 78 microsatellite loci identified in *Eucalyptus* spp. were evaluated for transfer to *Corymbia* spp. This set included 73 loci identified from a genomic library in *Eucalyptus grandis* (BRONDANI *et al.,* 2002; BRONDANI *et al.,* 1998) and 5 loci identified in a genomic library of *E. globulus* (MORAN *et al.,* 2004).

Transfer of *Eucalyptus* microsatellite loci was evaluated in two stages, firstly transfer to our laboratory and PCR conditions and secondly, transfer to the nonfocal taxa (i.e. species other than the species from which the microsatellite loci were isolated, in this study, *Corymbia* spp). Transfer was evaluated using a set of nine individuals, four grandparents, two parents and two offspring from the *Corymbia* mapping pedigree and an appropriate representative individual for the focal taxon. Markers were rated on five point scale where; 0 = not transferred to our laboratory i.e. PCR failed on focal and non-focal taxa, 1 = transferred to our laboratory i.e. PCR amplified in focal taxa only, 2 = transferred but segregation uncertain, 3 = transferred but not informative in mapping pedigree, 4 = transferred and mapped.

Microsatellite genotyping

PCR Amplification

A standardised PCR reaction was used for all microsatellite loci primer-pairs. The PCR buffer contained a final concentration of 20 mM Tris-HCl (pH 8.4), 50 mM KCl (supplied as 10 X PCR buffer, Invitrogen Life Technologies), 2.0 mM $MgCl_2$, 0.8 mM dNTP (total

Table 1. – Primer sequences and properties for 14 microsatellite loci isolated from *Corymbia citriodora* subsp. *variegata.*

Name	Oligo Sequence	Microsatellite repeat	T_m[1]	Allele size range[2] (bp)	PIC[3]
EMCRC 41	GACGGTAGCATTCTGTTCTTTGGCA	(GA)24	69.4	114-150	0.89
	TGGGTCGACTTTCAAGTCGCGT		70.5		
EMCRC 44	CCCCATCTCCATCCTCTG	(GA)24	61.5	188-246	0.28
	CGAACAACCCCCCATCT		61.8		
EMCRC 45	GCTAGGTTGGAACGAGCAAC	(GA)18	62.1	195-204	0.55
	TTGAGAAGAAGATGATATATAGCGTGA		61.5		
EMCRC 46	CCTTTGGAAGCATGCGTGT	(GA)13	64.6	151-202	0.20
	CCCTCCCTAAGTCCACATGA		62.3		
EMCRC 47	CCCACATAGAATCCCAACAT	(AG)22	59.5	185-217	0.89
	CTCGGTACCTCTGCGAAAC		60.8		
EMCRC 48	TGGAAAATGACATAGGCGAAA	(AG)7AT(AG)24	62.6	080-132	0.82
	CCGAACCAGAGAGTGGTG		60.2		
EMCRC 49	ACCAAGAAACGGGGAAAGAG	(GA)11(A)7(GA)2	62.6	251-286	0.89
	GTTTTGGAGAGAGGGGCAAG		63.4		
EMCRC 51	CTCGACCACCCGAGAGAA	(GA)15	62.5	250-277	0.90
	CACAGAATTCACTCCCTCCTAAA		61.7		
EMCRC 52	AGTCGAGAACGGCAACTCTG	(GA)18	62.9	132-163	0.87
	TCGCCCGTTTGCTTCTGT		65.3		
EMCRC 53	CGGTAGCATTCTGTTCTTTGG	(GA)25	61.9	101-155	0.88
	TTCTAGTCGCGTGAGTGTGG		62.5		
EMCRC 54	AAAGACACCAGCTTGACAACTTC	(GA)19	62	073-107	0.91
	CAATAATGTGTGAGCGTATTCCTG		62.8		
EMCRC 55	TGTAGATAAGGCGTGGAGAGG	(GA)21	61.5	270-297	0.91
	GCATACAATTATCCGCCAGAG		61.7		
EMCRC 56	TCCAAACCAAAACAACACCA	(GA)26	62.2	150-200	0.75
	GCTCTCCCCACAGCATAGA		61.4		
EMCRC 93	GCAACTCCAACGACAACAAC	(GA)16	61.6	131-166	0.87
	GGTCAATCTCCTCCACCAGTAA		62.6		

[1] Tm = Melting temperature of oligo determined by the thermodynamic method (RYCHLIK *et al.,* 1990).

[2] Allele size range based on 3 *C. torelliana* and 12 *C. citriodora* subsp. *variegata* individuals.

[3] PIC = polymorphism information content based on 12 *C. citriodora* subsp. *variegata* individuals.

dNTPs), 0.02 U/µl Platinum Taq (Invitrogen Life Technologies) and 0.2 µM each primer. Primers selected for mapping were resynthesised using a 5' fluorescent dye (6FAM, NED, VIC, or PET Applied Biosystems Foster City, CA or Sigma – Proligo, Lismore, Australia) for analysis using an Applied Biosystems 3730 Genetic analyser (Applied Biosystems). DNA templates were diluted to 0.4 ng/ul and a total of 2 ng was used in a total reaction volume of 13 µl.

Microsatellite loci were amplified using a series of touchdown programs with annealing temperatures (T_a) spaning 10 °C. The annealing temperature range was centred on the optimal Ta for each primer-pair. Optimal T_a were determined as 5 °C lower than the primer with the lowest melting temperature (T_m). Primer thermodynamic T_m were derived according to RYCHLIK *et al.* (1990) using the program Vector NTI (Informax, Frederick, MD USA) with parameters set to a primer concentration of 200 nM and a salt concentration of 50 mM. As an example, a primer-pair with an optimal Ta of 55 °C was assigned a program with an initial annealing temperature of 60 °C which decreased to 50 °C over 20 cycles, achieved by decreasing the temperature by 1 °C every second cycle. Cycling was composed of a denaturation step of a 95 °C hold for 1 min, a primer annealing step held at the annealing temperature for 1 min, then a primer extension step of 72 °C for 1 min. This was followed by a further 20 cycles using the final annealing temperature. Cycling was preceded by a hold at 95 °C for 5 min to provide a "hot start" and finished with a final hold of 3 min at 72 °C.

Marker scoring and Map construction

Marker loci were scored with the aid of GeneMapper V 3.5 software (Applied Biosystems). Grandparents and parents were genotyped for each marker to assist in identifying alleles. Once all markers had been scored, a marker phenotype rating of 1 to 3 was assigned to each marker based on its ease of scoring which was a function of allele separation and peak size.

Markers were coded for an outcrossing population for linkage analysis with JoinMap v 3.0 (Kyazma, Wageningen, The Netherlands) (VAN OOIJEN and VOORRIPS, 2001). Maps were prepared using the principles of framework and comprehensive maps; a framework map providing a map with a high confidence in grouping and order, whereas a comprehensive map reports linkages with a lower statistical support (KEATS *et al.,* 1991). Separate framework maps were prepared for each F_1 parent based on maternal and paternal data sets that contained loci informative for both parents as well as loci uniquely informative for the parent. Parental framework maps were generated with a grouping of LOD => 3 and with a interval support "Jump" value <= 2 (decrease in Chi-square goodness of fit measure that may accompany the addition of a locus to an order. A large jump value indicates a poor fit; values of 3–5 are considered generally applicable (VAN OOIJEN and VOORRIPS, 2001)).

A consensus (sex- and taxa- averaged) framework and comprehensive map were generated for the two F_1 individuals by integrating the two parental data sets into a single data set. Joinmap integrates data from various segregation types and recombination estimates from a variety of sources into a single map. Maps of corresponding parental homologues are integrated where a group includes markers informative for both parents. Non-homologous groups were identified by comparing maternal and paternal maps. Parental and grandparental data were excluded from the data set as Joinmap determines linkage phase automatically when calculating two-point linkage estimates. The framework consensus map was generated at grouping LOD => 4 and an interval support Jump value < 2. The comprehensive consensus map was obtained by "placing" "accessory" markers on to the framework map at the framework loci with the strongest linkage. Accessory markers were those that grouped with LOD support of at least 3 but could not be ordered at the stringent Jump value of less than 2. The comprehensive consensus map was prepared using MapChart 2.0 (VOORRIPS, 2001).

Genome length estimates

Genome lengths were estimated using the modification to Hulbert's method-of-moments approach (HULBERT *et al.,* 1988), method 3 as given in CHAKRAVARTI *et al.* (1991) and observed genome coverage as per NELSON *et al.* (1994). Separate data sets were created for each parent composed of markers informative for the respective parent and recoded fully informative markers. Fully informative (3 and 4 allele) markers were recoded as backcross markers for both parents (markers segregating with only two alleles in an intercross were excluded from the analysis because of the low amount of information they provided) and pair-wise recombination estimates obtained from Joinmap's "Strong linkages tabsheet" when grouped at required LOD (Z) value. Joinmap LOD values are based on a G^2 statistic for independence in a two-way contingency table, and are equivalent to standard LOD values where there is no segregation distortion in backcross markers (VAN OOIJEN and VOORRIPS, 2001).

Results and Discussion

Microsatellite markers developed de novo in subsp. variegata and transferred to C. torelliana

Primer-pairs for 14 newly developed microsatellite loci isolated from subsp. *variegata* amplified products of around the expected size in a test panel of subsp *variegata* and *C. torelliana* (data not shown). Polymorphism information content for the markers ranged from 0.2 to 0.91 with an average of 0.79 ± 0.24 for a set of 12 subsp. *variegata* individuals (*Table 1*). This average was comparable to the gene diversity for subsp. *variegata* assessed by microsatellites previously developed *de novo* from this taxa (av. H_o = 0.86 for 7 loci; (KING, 2004)) and with gene diversity based on microsatellites in other widespread eucalypts (BRONDANI *et al.,* 1998; STEANE *et al.,* 2001). Out of the 28 markers developed from subsp. *variegata*, 27 segregated in the mapping cross and tended to have high information content for mapping with 17 out of 28 loci segregating in both parents and having highly informative mating configurations with 3 or 4

Table 2. – Amplification and transfer characteristics for *Eucalyptus* microsatellite loci in *Corymbia* sp.

Source (Species/Library type)	No transfer to Lab. (0)[1]	Focal taxa only (1)	Transfer but uncertain seg. (2)	Not informative (3)	Mapped (4)	Total
E. grandis - G	32	4	1	6	30	73
E. globulus - G	2	0	0	0	3	5
Total	34	4	1	6	33	78

[1] See methods for full description of classes.

segregating alleles (data not shown). Surprisingly, null alleles occurred at fewer loci (4) in *C. torelliana* parents of the mapping cross than subsp. *variegata* parents (5) suggested relatively little sequence divergence in priming sites between the two taxa. This was consistent with recent data from internal transcribed spacers (ITS) which suggested *C. torelliana* (Section Cadagaria) may be closer to the spotted gums (Section Politaria) than previously thought (Pers. Comm. J Ochieng). Based on 302 bp of ITS1 and the 5.8 subunit, there was only 2 bp substitutions between *C. torelliana* and subsp. *variegata*, yet, 5 and 6 bp substitutions were found amongst members of the Politaria. All loci contained $(GA)_n$ repeat types and were perfect except for EMCRC 48 and 49 (*Table 1*).

Transfer and conservation of microsatellite loci from Eucalyptus

A set of 78 microsatellite markers identified in *Eucalyptus* species were tested for transfer to *Corymbia*. Microsatellites were derived from two different species, 73 were identified in a genomic library of *E. grandis* (BRONDANI *et al.,* 2002; BRONDANI *et al.,* 1998) and 5 from a genomic library of an *E. globulus* individual (MORAN *et al.,* 2004). A total of 44 out of the 78 loci were amplifiable under the conditions used in this study (based on amplification of a PCR product of expected size from a representative of the focal taxa) (*Table 2*). Amongst loci that were amplifiable under the standardised conditions used in this study, 90% of loci transferred from *Eucalyptus* to *Corymbia* with more or less equal transfer to *C. torelliana* and subsp. *variegata* (*Table 2*). Although the sample size was small for *E. globulus*, transfer from *E. grandis* (90%) to *Corymbia* sp appeared more successful than *E. globulus* (60%). A review of cross trans-

Table 3a. – Map parameters for *Corymbia* genetic maps.

Map	No. Markers in Dataset	LOD Grouping	No. Mapped Markers	No. Groups	Map Length cM K
Maternal Frame.	54	3	42	13	399
Paternal Frame.	60	3	46	13	489
Consensus Frame.	64	4	47	13	367
Consensus Comprehensive	64	3	45+9[1]	12	417

[1] Comprehensive map was based on a framework set of markers + accessory markers.

Table 3b. – Genome length and physical equivalent estimates for *Corymbia*.

Map	Grouping criteria (LOD)	No. Locus pairs	Max. Dist.	Meioses	Expected genome Length[1] (cM K)	Obs. No. Groups + Unlinked (L)	Obs. Coverage (cM K) and (%)[2]	1C physical equivalent (kbp)[3]
Symbol	Z	K	X	M	E(G)	L	C_o	
Mat.	3	47	35	1081	1610	10+12	784 (49)	236
	4	39	31	1081	1719	9+16	833 (49)	221
	5	36	27	1081	1622	9+16	696 (43)	234
	11	20	15	1081	1622	1+42	879 (54)	234
Av.					**1643**		**798 (48)**	**231**
Pat.	3	58	35	1378	1663	10+12	874 (52)	228
	4	49	30	1378	1687	10+14	879 (52)	225
	5	46	24	1378	1438	10+15	853 (55)	264
	11	17	13	1378	2108	5+32	827 (39)	180
Av.					**1724**		**858 (50)**	**225**

[1] Genome Length = E(G) = 2*MX/K Hulbert's method-of-moments approach with Chakravarti method 3 modification (CHAKRAVARTI *et al.,* 1991).

[2] Observed Map coverage, $C_o = G_f + X(L\text{-}R)$ where R = 11, the haploid chromosome number in *Eucalptus* and the G_f for the maternal and paternal maps were 399 and 489 cM K respectively.

[3] Genome sizes used to estimate physical equivalents were taken from GRATTAPAGLIA and BRADSHAW (1994). Estimate for *Corymbia* hybrid genome size was 380 Mbp, the average of *Corymbia torelliana* and *Corymbia maculata*.

ferability of microsatellites in a wide range of angiosperms and gymnosperms concluded that 30–40% was typical for intergeneric (ROSSETTO, 2001), therefore our results suggest *Eucalyptus* and *Corymbia* are relatively close genera.

Although transfer may be high between the two genera, the information content of transferred loci may be lower than those identified *de novo* as only 30% of loci sourced from *E. grandis* and 20% from *E. globulus* where of the most informative mating configurations

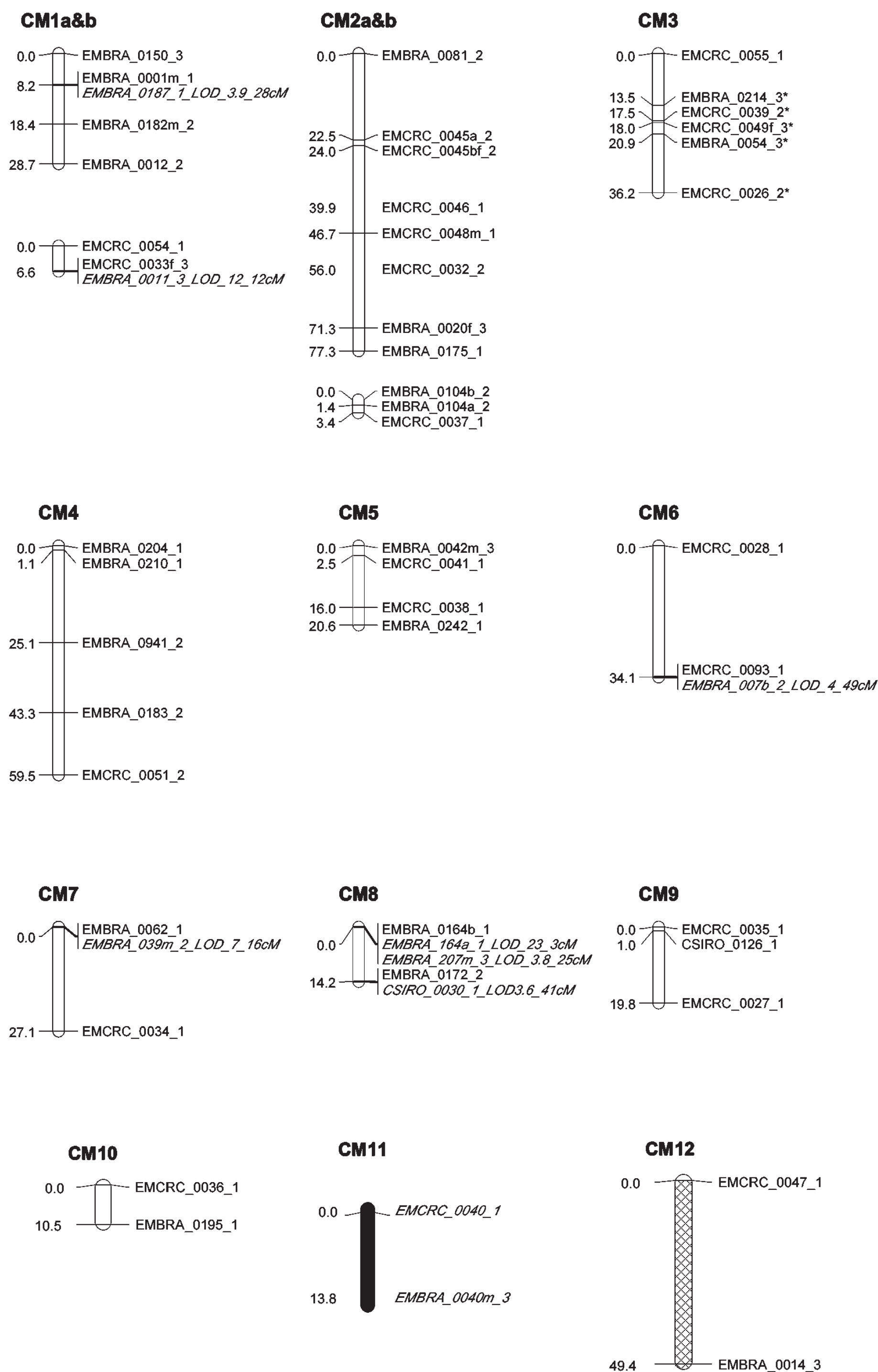

Figure 2. – A comprehensive consensus map for two *Corymbia torelliana* x *citriodora* subsp. *variegata* F_1 hybrids. The total map distance was 417 cM Kosambi, 47 framework and 9 accessory markers formed 12 Groups (LOD =>4; except CM12 (LOD 3)). Accessory markers are italicised and are reported at the framework loci that they were most strongly linked with their LOD and pair-wise distances. Groups CM11 and CM12 consisted of markers that segregated uniquely in the paternal or maternal parent respectively, and are highlighted with different shading. Groups CM1a&b and CM2a&b are linked at LOD 3 – indicated as separate groups but aligned vertically. Markers exhibiting segregation distortion are indicated by an asterisk ($\alpha \leq 0.05$). A marker quality rating (1 to 3) was suffixed to each loci, with 1 indicating the highest quality (see methods).

with 3 or 4 segregating alleles in the mapping cross (data not shown).

Divergence in priming site sequence as indicated by the occurrence of null alleles, was concordant with phylogenetic relationships. Overall the number of loci with null alleles was high (70%), with markers transferred from *Eucalyptus* showing a higher rate of null allele occurrence (73%) than markers that were identified in subsp. *variegata* (42%). A high proportion of nulls was expected in this mapping population both because of the width of the cross, and the expected divergence in priming site sequence between *Eucalyptus* and *Corymbia*.

Parental maps

The maternal framework map had a total length of 399 cM Kosambi (K), was comprised of 42 linked markers in 13 groups, with an average map interval of 9.7 cM K (*Table 3a*; Map not shown). The paternal framework map had a total length of 489 cM K, was comprised of 46 linked markers in 13 groups, and had an average map interval of 10.6 cM K (*Table 3*; Map not shown).

As expected for two maps based on largely overlapping data sets, synteny and colinearity was extensive. The two maps were entirely collinear; however there were two instances were synteny was imperfect, largely due to markers segregating uniquely in the maternal or paternal datasets. Maternal and paternal homologues were identified for all groups except one pair in the paternal map (Group 10; EMBRA0040m & EMCRC 0040) and Group 2a (EMCRC0054, EMCRC0033f, EMBRA0011) on the maternal map. Minor differences in multi-locus map distances were also observed between the maps where parent-specific markers mapped uniquely. Missing data was relatively low (1.9%) for the total dataset (90 F_2 offspring plus the two F_1 parents and 4 grandparents); the average number of missing data per marker was 1.7 ± 3.2 (SD).

Genome lengths were estimated for each parental map at a range of grouping values (LOD 3, 4, 5 and 11; *Table 3b*). The genome length estimates for the maternal parent were stable over a range of LODs with an average value of 1643 cM K. Estimates of genome lengths for paternal maps were more variable, but on average, larger (1724 cM K) than the maternal map. The map coverage for the maternal and paternal genomes was 48 and 50%, respectively (*Table 3b*).

Meiotic rates of recombination in pollen and ovules differ for many plants (KOROL *et al.,* 1994). In *Pinus*, recombination rates for pollen can be as much as 43% higher than for ovules (LEDIG, 1998; MORAN *et al.,* 1983). This is thought to be adaptive and related to the dispersal mechanism for seed and pollen in pines, as pollen is dispersed much more widely than seed and is likely to encounter much greater environmental heterogeneity. Higher recombination rates in male meiocytes may provide a greater chance of the recombinant offspring being preadapted to the divergent environment that it must survive in. In contrast, *Eucalyptus* maternal and paternal maps tend to be similar in length (e.g. BUNDOCK *et al.,* 2000; MYBURG *et al.,* 2003). The differential in dispersal distances for pollen and seed in eucalypts is much lower than pines. *Eucalypts* are animal pollinated with most pollen distributed within 200 m. Seed dispersal is passive in eucalypts, but may be aided by wind and is usually limited to a distance equal to the tree height (< 100 m) (HOUSE, 1997; POTTS *et al.,* 2003). *Corymbia torelliana* is an exception as seed may be dispersed by native bees over distances of around 300 m (WALLACE and TRUEMAN, 1995).

Dispersal mechanisms for *Corymbia*, as with *Eucalyptus*, suggest male and female meiotic recombination rates may be similar. The average genome length estimates for *Corymbia* male and female meiocytes were similar (< 5% difference). The larger estimate for the male genome may be due to a lower estimate accuracy at LOD 11 because the small sample size of meioses approaches a lower limit for reliability (CHAKRAVATI *et al.,* 1991). The estimates are also based on F_1 hybrids and therefore potentially confounded by differences in recombination rates between parental taxa and recombination shrinkage that may occur in hybrids (KOROL *et al.,* 1994; STEBBINS, 1971).

Consensus maps for Corymbia sp

A framework consensus map was constructed from a single dataset of 64 segregating loci (see methods; *Table 3a*; map not shown). The framework consensus map (LOD =>4) was based on 47 linked markers in 13 groups, with a total map distance of 367 cM K and average map interval of 8 cM K (*Table 3a*). The shorter map distance for the framework map compared to the parental maps was a consequence of the higher statistical support applied to grouping and ordering that excluded some markers from the framework consensus map. A comprehensive consensus map was also constructed to report linkages with lower statistical support (Grouping LOD => 3 and Jump values > 2) (*Figure 2* and *Table 3a*). The comprehensive consensus map consisted of 12 groups with a total distance of 417 cM K and was based on 54 markers, including 9 "accessory" markers that were placed onto the framework map of 45 markers. The total map length of the comprehensive map was longer than the framework map because parent-specific groups were pooled into one map (i.e. groups consisting of markers which were informative for only one of the parents, Groups CM 11 and CM 12). The length of the comprehensive map may be slightly inflated, therefore, because these groups may be homologous, but without markers informative for both parents on these groups it was not possible to determine whether they should be integrated or not. Accessory markers were loci that could not be ordered at "Jump" <=2 on the framework maps but nonetheless grouped at LOD > 3 (EMBRA 0011, EMBRA 007b, EMBRA 039, EMBRA 164a, EMBRA 187, EMBRA 207, CSIROEg 030), and a pair of markers that formed a group at LOD 3 (Group 11; EMCRC 0047 and EMBRA 0014). In addition, on the comprehensive map, two pairs of groups that were not linked at LOD 4 on the framework map, grouped at LOD 3. This linkage was indicated by aligning the pairs vertically (*Figure 2* Groups CM1a&b, CM2a&b). Group CM1a&b grouped at LOD 4 but could not be ordered on the framework consensus map, whereas Group CM2a&b grouped at LOD 3 but could not be ordered on the paternal framework map. Eleven loci were unlinked

Table 4. – Group 3 on the *Corymbia* map contains a region where there was a deficit of one class of hybrid offspring genotypes in the F_2 generation (in bold). All markers were distorted at p-value =< 0.05.

Marker	*Grandparental genotypes*[1]				*Mat. F_1*	*Pat. F_1*	*Offspring genotypes*			
	T1T1	V1V1	T2T2	V2V2	H	H	(T1T2)	(T2V1)	(T1V2)	(V1V2)
EMBRA 214	112/N	110/N	110/N	113/N	112/N	113/110	11	25	30	22
EMCRC 039	N/N	193/N	N/N	207/N	193/N	207/N	28	**11**	24	26
EMBRA 054	158/N	161/169	158/163	161/N	169/N	161/N	26	**10**	26	28
EMCRC 026	N/127	113/137	111/N	105/119	137/N	105/N	19	**13**	36	21

[1] e.g. T_1T_1 symbolises the grandparental genotype for *C. torelliana* grandparent 1. Genotypes reported as allele sizes in base-pairs. N = null allele.

(CSIROEg 094, EMBRA 008, EMBRA 028, EMBRA 1056, EMBRA 145, EMBRA 155, EMBRA 007b, EMCRC 31, EMCRC 44, EMCRC 52, EMCRC 53).

Hybrid inviability

Marker segregation distortion is a difference in the observed offspring genotype class frequency from that expected based on Mendelian inheritance. The number of markers showing distortion was higher than expected due to sampling error (10 loci in a set of 64 markers p-value = <0.05). All five of the distorted markers that mapped clustered onto Group 3 (*Figure 2*). All five markers were deficient in an allele inherited from the maternal parent. Furthermore, at three of the loci (EMCRC 039; EMBRA 054 and EMCRC 026), there was a deficit in one hybrid genotype class (one locus had a backcross configuration and therefore hybrid and parental genotypes could not be distinguished; and the other EMBRA 214 did not fit the pattern, it was deficient in the *C. torelliana* homozygote (T_1T_2) offspring genotype) (*Table 4*). In particular, there was a deficit of F_2 genotypes with an allele from the maternal F_1's subsp. *variegata* parent and an allele from the paternal F_1's *C. torelliana* parent.

Clustering of distorted markers where there is a directional pattern, suggests a biological rather then methodological explanation for marker distortion (KEARSEY and POONI, 1996). Hybrid dysgenesis and segregation distortion have been features of mapping experiments involving wide crosses in plants, and they tend to be the rule for studies of interspecific hybrids (KOROL *et al.,* 1994; RIESEBERG and LINDER, 1999). Although there may be several methodological or biological explanations for segregation distortion, in our case, hybrid incompatibility would appear to be a likely explanation as there is a deficiency of one hybrid genotypic class. The location of a hybrid inviability locus in the distorted region within Group 3 would account for distortion in linked markers. Moreover, as only one of the hybrid classes was deficient, it suggested a specific interaction between two grandparental backgrounds rather than a more general incompatibility.

Hybrid inviability and advanced generation hybrid breakdown is a key issue for breeders developing eucalypt hybrids and generally relates to the taxonomic distance between the parental species (POTTS and DUNGEY, 2001). High mortality (i.e. 50%) has been noted in outbred F_2 populations of *Eucalyptus globulus* where the grandparents crossed to produce F_1 were from divergent provenances (FREEMAN, 2005). High levels of abnormals (41–47%) and low germination (57–61%) were also reported for backcrosses between *E. grandis* and *E. globulus*, species from different sections of the *Symphyomyrtus* series (GRIFFIN *et al.,* 2000). In a detailed study of hybrid breakdown, genome wide surveys of the parents of *E. grandis* x *E. globulus* backcrosses indicated that in eucalypts, complex multi-genic interactions where likely to account for most post-zygotic isolation (MYBURG *et al.,* 2004).

Based on the experiences with other eucalypt hybrids, the intersectional *Corymbia* hybrid used in this study would be expected to exhibit pronounced hybrid inviability. There is evidence that both the F_1 and F_2 exhibit hybrid inviability, although without direct comparative data, it is unclear whether the inviability is more extreme in the F_2 (i.e. hybrid breakdown). Low germination (50%) and survival of the F_2 hybrid family (76% and 50% of germinants survived at 6 and 14 mths respectively) in this study were congruent with hybrid incompatibility. Hybrid inviability was believed to account for high levels of abnormals and mortality in F_1 families in the nursery as well as a higher variability in early field growth compared to parental taxa (DICKENSON *et al.,* 2004). In a trial comparing early growth and survival of F_1 with parental taxa, F_1 families experienced significantly higher mortality at age 3.5yrs (t test for independent samples two-tailed p-value = 0.017; av ± SE F_1 55 ± 4.0 n = 5; parental families 78 ± 6.5 n = 5; from *Figure 3* in DICKINSON *et al.* (2004)).

Comparative analysis

The *Corymbia* map was compared to a map for *Eucalyptus grandis* (EM) to evaluate the extent of synteny

Table 5. – Comparative analysis of a *Corymbia* map (CM) with a *Eucalyptus* sp. map (EM) (BRONDANI *et al.,* 2002).

Name	*CM Group*	*EM Group*
EMBRA 001	1	8
EMBRA 011	1	1
EMBRA 012	1	1
EMBRA 020	2	7
EMBRA 054	3	5
EMBRA 042	5	7
EMBRA 007	6	9
EMBRA 039	7	11
EMBRA 062	7	11
EMBRA 040	11	10
EMBRA 014	12	8
EMBRA 008	Unlinked	6
EMBRA 028	Unlinked	6

Table 6. – Genome length, size and physical equivalents for *Eucalyptus*.

Species	Sex	Genome Length (cM K)	Grouping criteria LOD	Obs. Map cM (K)	Genome Size (Mbp/1C)	1C physical equivalent (kbp)	Reference
E. grandis	Maternal	1620	5	1552	641	395.7	(GRATTAPAGLIA & SEDEROFF 1994)
E. urophylla	Paternal	1156	5	1101	645	558.0	(GRATTAPAGLIA & SEDEROFF 1994)
E. grandis	Maternal	1331	11	1335	641	481.6	(MYBURG *et al.* 2003)
E. globulus	Paternal	1273	11	1405	530	416.3	(MYBURG *et al.* 2003)
E. grandis x E. globulus F1	Paternal	1469	11	1448	-	-	(MYBURG *et al.* 2003)
E. grandis x E. globulus F1	Maternal	1411	11	1318	-	-	(MYBURG *et al.* 2003)
E. globulus	Maternal	1133	4.9	701	530	467.8	(BUNDOCK *et al.* 2000)
E. globulus	Paternal	1277	4.9	1013	530	415.0	(BUNDOCK *et al.* 2000)

and colinearity (BRONDANI *et al.,* 2002). Moderate synteny was observed between the *Corymbia* and *Eucalyptus* maps. Instances of incongruence in synteny were associated with weak linkage and therefore may be due to incomplete map coverage on the *Corymbia* map (CM). *Corymbia* homeologues were identified for eight *Eucalyptus* groups (*Table 5*). Thirteen markers were mapped on both maps but only 11 of them were grouped on the *Corymbia* map (*Table 5*). This included 5 pairs of syntenic markers from the *E. grandis* map (EM Group 1 EMBRA011 & EMBRA 012; EM Group 6 EMBRA 008 & EMBRA 028; EM Group 8 EMBRA 001 & EMBRA 014; EM; EM Group 7 EMBRA 020 & EMBRA 042; Group 11 EMBRA 062 & EMBRA 039). Two of the five pairs were syntenic on both maps (EM Group 1 and CM Group 1; EM Group 11 & CM Group 4). The linkage between EMBRA 011 & EMBRA 012 was weak on both the CM (pairwise rf 0.39 LOD 1.78) and EM. Linkage between these two markers was also weak on a map for a second *Eucalyptus* sp., *E. globulus* (Multipoint estimate 84.4 cM (K) (FREEMAN, 2005); (pairwise rf 0.48 LOD 0.07; Pers. Comm. J Freeman). No map distance was available for EMBRA 062 & EMBRA 039 to compare the estimate from the CM (they were mapped to parental homologues which could not be merged on the EM). In the three instances of non-synteny, marker pairs were loosely linked on the EM (i.e. > 42.6 cM (K)). Absence of synteny on the CM therefore may be due to low map coverage. There were no instances where colinearity could be examined (i.e. 3 or more markers linked together on the two maps). Increased power to test *Corymbia-Eucalyptus* synteny must await more extensive map coverage in both taxa.

Genome length estimates for *Eucalyptus* sp have tended to be similar to or smaller than our estimates for *Corymbia* (*Table 3b* and *Table 6*). Estimates of physical map equivalents for *Corymbia* tended to be smaller (180–264 kbp/1C) than *Eucalyptus* sp (395–558 kbp/1C), because of the smaller genome size of *Corymbia* sp. (GRATTAPAGLIA and BRADSHAW, 1994).

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Short Note: High Throughput Microsatellite Genotyping in Oak Species

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Abstract

Microsatellites are widely used markers for multiple purposes in oaks. We describe a complete procedure for cheap DNA extraction and fast microsatellites genotyping by multiplex PCR. 10 loci were selected to form two multiplex kits including three loci that show a high differentiation between *Quercus robur* and *Q. petraea*. The loci were tested in three oak species and show a high mean genetic diversity of 0.84. The cumulative exclusion probability for parentage analysis was 0.999977 for single parent and 1.0 for paternity. Finally, the relatively high differentiation coefficient (Gst = 0.04) will facilitate species assignment based on genotypes in oaks.

Key words: microsatellites; multiplex PCR; *Quercus*; genetic assignment; parentage analysis.

Oaks are common species in a large part of the world and are involved in an increasing number of genetic studies concerning especially population genetic structure and gene flow. New powerful methods for analysing multilocus genetics data allow inference about past demographic events, genetics assignment or detection of selection (CHIKHI and BRUFORD, 2005). We took advantage of technical laboratory progresses to increase availability of multilocus microsatellites data. Combining multiplex PCR and automated capillary sequencer speed up the genotyping compared to silver straining gel or gel plate automated sequencer. In the same time it decreases cost of genotyping and allows to reach high loci number analysis which is needed for high resolution studies in natural populations. In this short note, we present a protocol that allows fast DNA extraction and genotyping for 10 microsatellites in two multiplex PCR.

We used a modified protocol based on CTAB/dichloromethane (DUMOLIN et al., 1995) allowing DNA extraction in 96 wells plates format. This modification provides high quality DNA extraction from 192 individuals in one day at a low cost compared to commercial kits. One centimeter square of leave or three to five buds are cut into small pieces and put in a 1.2 mL microtube (Qiagen, Cat. No. 19560) with two 2 mm steel balls, a small amount of insoluble PVP and alumina, 100 µL of extraction buffer (CTAB 20 mg.mL^{-1}, EDTA (pH 8) 0.02M, Tris HCl (pH 8) 0.1M, NaCl 1.4M, soluble PVP 10 mg.mL^{-1}). Vegetable tissues and 300 µL of extraction buffer are ground in a Retsch-Mill at 30 vibrations per second for 1 min for each side. The microtubes are incubated at 55 °C for 1 hour in a vertical position with shaking. After cooling, 300 µL of dichloromethane is added and microtubes are shaken. After 20 minutes of centrifugation at 6200 rpm and 4 °C, the upperphase is transferred in new microtubes, 300 µL of cold isopropanol is added and after shaking the microtubes are placed at –20 °C for 1 hour in a vertical position. After a centrifugation step of 20 minutes at 6200 rpm and 4 °C, the supernatant is removed, 500 µL of ethanol is added and microtubes are shaken before centrifugation for 20 minutes at 6200 rpm and 4 °C. Supernatant is then removed and the pellet is dried in a vacuum system concentrator for 15 minutes at 55 °C. Finally the pellet is resuspended in 50 µL of pure water. To avoid lost of DNA pellet when removing the upperphase after each centrifugation step, microtubes are kept on ice.

We selected microsatellites loci that are highly differentiated between *Quercus robur* and *Q. petraea* (SCOTTI-SAINTAGNE et al., 2004; P. Goikoetxea, unpublished data). Before multiplexing, primers were checked for primer-primer interaction using the AutoDimer software (VALLONE and BUTLER, 2004). One set, called KIT1, comprises the five following loci QrZAG11, QrZAG39, QrZAG96, QrZAG112 and QpZAG110 and the second one, five others (KIT2): QrZAG5, QrZAG7, QrZAG20, QrZAG65 and QrZAG87 (KAMPFER et al., 1997; STEINKELLNER et al., 1997). On the 10 selected microsatellites, 7 are unlinked (*Table 1*). Each forward primer was marked with a fluorescent dye (MWG and Applied Biosystems) as shown in *Table 1*. PCR were carried out in a final volume of 15 µL including 5 µL of 1/50 to 1/200 diluted DNA, depending on DNA concentration

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