CIRAD

FORÊT

PROGRAMME ARBRES ET PLANTATIONS

RAPPORT DE MISSION

en TASMANIE

Du 31 janvier au 10 février 1999

Daniel Verhaegen

Mars 1999

OBJET DE LA MISSION.

Cette mission a été planifiée afin que le programme Arbres et Plantations du CIRAD Forêt puisse participer au Symposium intitulé " Molecular Genetics of Eucalyptus". Ce symposium était organisé à Hobart (Tasmanie) les 4 et 5 février, par le Cooperative Research Centre for Sustainable Production Forestry (CRCSPF). Les présentations orales ont été faites à l'Université de Tasmanie. De nombreux laboratoires australiens et des laboratoires externes (Afrique du Sud, Brésil, Ecosse et France) étaient représentés, quelques étudiants ont assisté aux présentations et aux discussions.
Les principaux laboratoires menant des recherches sur la biologie moléculaire des *Eucalyptus* sont à une étape importante. Les recherches en cours sont :

- ➥ généralement redondantes : distances génétiques, cartes génétiques, détection de QTL (Quantitative Trait Locus), Sélection Assistée par Marqueurs (SAM)...
- ➥ parfois spécifiques : ADN chloroplastique, gènes candidats de la lignine ou de la cellulose...
- ➥ non reliables : marqueurs différents, espèces différentes...

La nécessité d'établir une coopération internationale était déjà apparue durant le congrès IUFRO au Brésil, en 1997. A la fin de ce symposium, une réunion a été organisée sur la demande des chercheurs français et brésiliens afin d'étudier ensemble les possibilités de coopération entre nos différents laboratoires.

Plusieurs objectifs étaient donc visés par cette mission :

① Présenter aux chercheurs utilisant les marqueurs moléculaires sur les *Eucalyptus,* les travaux que mènent le programme Arbres et Plantations du CIRAD Forêt dans ce domaine.

② Participer à la table ronde qui était organisée sur invitation pour un nombre limité de chercheurs. Les chercheurs invités étaient :

Margaret Byrne (CALM, Australie), Dario Grattapaglia (EMBRAPA, Brésil), David Marshall (SCRI, Ecosse), Gavin Moran (CSIRO, Australie), Brad Potts (CRCSPF, Australie), Mervyn Shepherd (CRCSPF, Australie), René Vaillancourt (CRCSPF, Australie), Daniel Verhaegen

(CIRAD-Forêt, France).

Invités de dernière minute : Gerd Bossinger (CRCHFPS, Australie), Chris Harwood (CSIRO, Australia), Celso Marino (UNESP São Paulo, Brésil), et Brenda Wingfield (Forestry and Agricultural Biotechnology Institute, Department of Genetics, University of Pretoria South Africa).

Invités absents : Christina Marques (RAIZ, Portugal), Bob Teasdale (FORBIO, Australie).

③ Profiter du passage au CRCSPF à Hobart pour visiter le laboratoire de biologie moléculaire de René Vaillancourt et discuter du programme d'amélioration génétique de *E. globulus* développé par Brad Potts. A notre demande et aussi pour Alix Pernet (qui vient d'être recrutée à l'AFOCEL), une visite de terrain a été organisée après le Symposium. Cette visite organisée par Brad a été l'occasion de voir différents dispositifs de terrain et de visualiser l'aire naturelle des E. gunii introduits en France. Cette espèce semble en péril car nous avons observé une forte mortalité des arbres et une absence de régénération naturelle. La visite d'une compagnie privée (North Forest Products) a été organisée dans le Nord de la Tasmanie.

PERSONNES RENCONTRÉES LORS DE CETTE MISSION.

Prof	Reid	Jim	Directeur CRCSPF Cooperative Research Centre for Sustainable Production Forestry	Hobart Tasmanie
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Dr	Bossinger	Gerd	CRCHFPSSF Cooperative Research Centre for Hardwood Fibre and Paper Science School	Melbourne Australie
Dr	Byrne	Margaret	CALM Science Department of Conservation and Land Management	Western Australia
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Prof	Ferraz do Valle	Celina	Votoratim Celulose e Papel S.A.	Sao Paulo Brésil
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Prof	Wingfield	Brenda	Forestry and Agricultural Biotechnology Institute. Department of Genetics. Université de Pretoria	Afrique du Sud

La liste complète des participants au Symposium "Molecular Genetics of Eucalyptus" est donnée en Annexe 1.

PRINCIPAUX RÉSULTATS PRÉSENTÉS PENDANT LE SYMPOSIUM.

Le programme qui a été proposé pendant ce Symposium, concernait la phylogénie, la structure des populations, les flux génétiques, la structure du génome, la détection de QTL. Les résumés des présentations orales sont donnés en Annexe 2 et les résumés des posters sont donnés en Annexe 3.

La première journée a été entièrement consacrée à la phylogénie , à la structure des populations, la diversité génétique et aux flux de gènes. Les différentes études de phylogénie aboutissaient toutes à des séries de dendrogrammes qui ne "remettaient pas en cause" la classification des *Eucalyptus*. Seules les études concernant l'ADN chloroplastique perturbent ces classifications. Ces marqueurs

permettent de faire l'hypothèse que l'hybridation à un impact fort sur la différenciation des espèces. Les différents haplotypes étudiés sur *E. globulus* montrent que le Sud de la Tasmanie a été une zone refuge de l'espèce pendant l'ère glaciaire. Les mutations observées montrent que l'espèce remonte progressivement vers le Nord de la Tasmanie depuis la fin des glaciations.
L'analyse de la variabilité génétique des populations d'amélioration d'*E. urophylla* et *E. grandis* au Brésil avec les marqueurs RAPD montre que certaines populations ont des bases génétiques très étroites. Des variabilités élevées peuvent cependant être trouvées chez *E. grandis*.
Les marqueurs microsatellites ont été utilisés pour étudier les flux de gènes dans des vergers à graines d'*Eucalyptus* en Amérique du Sud. Avec *E. grandis* un grand nombre d'arbres contribuent comme parents mâles dans l'observation de la descendance d'un arbre. Les arbres efficaces ne sont pas les plus proches voisins. Les résultats présentés sont très proches des résultats obtenus à Bordeaux sur le chêne (le pollen efficace vient parfois de plus de 300 m).

La seconde journée a été consacrée à la structure du génome des *Eucalyptus* et à la détection de QTL. Des exposés très généraux sur les différentes utilisations du marquage moléculaire ont été présentés par Dario Grattapaglia et Gavin Moran.
Une étude montre la possibilité d'établir les synténies entre les cartes génétique *Eucalyptus globulus*, *Eucalyptus urophylla* et *Eucalyptus grandis*. Parmi les 20 marqueurs SSR publiés pour ces deux dernières espèces, 7 ont été positionnés sur les cartes génétiques d'*E. globulus* établissant ainsi l'intérêt d'intégrer ces marqueurs pour l'établissement de cartes consensus.
Une région du génome de l'*Eucalyptus grandis* semble contrôler la production de limonène (huile prédominante dans les feuilles).
Deux familles de pleins frères ayant un parent commun ont été utilisées avec des marqueurs co-dominants (RFLP et SSR) pour détecter des QTL d'enracinement *in vitro*. La faible répétabilité du pourcentage d'enracinement *in vitro* ne permet pas de conclure sur la validité des QTLs.
Les ADNc isolés à partir du cambium des *Eucalyptus globulus*, sont parfois spécifiques aux *Eucalyptus* ou mettent en évidence des gènes connus pour leurs activités méristématiques, la prolifération cellulaire, la morphologie des fibres, la teneur et la composition des lignines, la biosynthèse de la cellulose, les caractéristiques des parois cellulaires, la synthèse d'hormones etc ...
Coup de bluff habituel ou part de vérité ? FORBIO a annoncé sic : "réaliser le séquençage intégral du

génome des *Eucalyptus*"

RECHERCHES SUR LES *EUCALYPTUS* DU PROGRAMME ARBRES ET PLANTATIONS.

L'exposé qui a été fait à l'Université de Hobart visait à présenter les différentes activités du laboratoire de génétique du CIRAD Forêt à Baillarguet. Deux domaines ont été approfondis :
1 l'application de la sélection assistée par marqueurs actuellement mise en place dans les dispositifs d'essais du Congo, les études sur la stabilité des QTL dans le temps et dans différents fonds génétiques.
2 l'approche sur la recherche de gènes candidats actuellement développée à travers le projet biotechnologies. Pour la première fois des résultats montrant la co-localisation entre le positionnement de gènes de fonction connue sur les cartes génétiques et le positionnement de QTL pour des caractères d'intérêt économiques ont été présentés. Ces résultats ont soulevé un grand intérêt de la part des différents chercheurs présents :

soit directement : Dario Grattapaglia (EMBRAPA) souhaite obtenir la séquence du gène EgPar car lui aussi trouve un QTL de bouturage chez *E. urophylla* ; il a aussi souhaité connaître notre approche avec ces gènes candidats au niveau technique (méthode SSCP), et au niveau scientifique (test de la variabilité existante sur l'ensemble de notre population d'*Eucalyptus* et contrôle de la relation avec l'aptitude au bouturage). Karen Thamarus (CSIRO) a demandé s'il était possible d'obtenir les séquences des gènes de la voie de biosynthèse des lignines.

Soit indirectement : Gavin Moran a demandé à Gillian Rasmussen de la compagnie North Forest Products de m'interroger sur nos techniques et nos résultats, seulement lors de ma visite à la compagnie qui était prévue après le Symposium. Il faut préciser ici que Gavin Moran avait présenté l'intérêt des gènes candidats de la lignine mais sans citer ni les gènes qu il étudie, ni les résultats obtenus. L'exposé du CIRAD a donc fait plaisir à un certain nombre de participants (essentiellement ceux du public qui publient leurs résultats).

De nombreuses discussions ont également eu lieue avec Brad Potts et René Vaillancourt concernant notre papier sur les distances génétiques. Un échange par mail de fichiers et d'analyses pourrait être à l'origine d'une collaboration plus intense.

Photo 1 : Quelques membres du groupe Eucalypt Genome Initiative.
De gauche à droite : Dario Grattapaglia, Daniel Verhaegen, Celso Marino, Mervyn Shepherd, Brad Potts, René Vaillancourt, Celina Ferraz do Valle.
Assis : Margaret Byrne et David Marshall.

COLLABORATION INTERNATIONALE SUR LE GÉNOME DES *EUCALYPTUS*.

La table ronde qui était organisée après le Symposium a été dirigée par Jim Reid Directeur du CRCSPF. Les différents points discutés lors de cette table ronde sont présentés en annexe 4. L'objectif principal des discussions était d'obtenir un accord de principe de chacun des participants sur l'importance de regrouper les informations des nombreuses cartes génétiques et de renforcer nos collaborations dans certains domaines. L' idée principale qui émergeait était d'obtenir des cartes génétiques consensus pour les principales espèces suivantes : *E. globulus*, *E. nitens*, *E. urophylla* et *E. grandis*. Plusieurs buts étaient visés lors de cette réunion :

* de créer un groupe de travail sur la biologie moléculaire des *Eucalyptus*,
* trouver un thème de recherche pour démarrer des collaborations plus étroites entre les différents laboratoires : CSIRO, Universités d Australie, CRCSPF, EMBRAPA, RAIZ, SCRI, CIRAD,
* établir une codification commune pour uniformiser nos données,
* d'étudier les possibilités de monter des projets communs avec une recherche de financement.

En début de séance Dario Grattapaglia a invité les chercheurs (ce message s'adressant principalement à Gavin Moran, à David Marshall et à Margaret Byrne) ayant développés les marqueurs microsatellites à publier rapidement leurs résultats. Car son équipe publiera avant la fin de l'année plus de 200 séquences pour chacune des deux espèces qui nous concerne (*E. urophylla* et *E. grandis*). Par la suite, il sera donc difficile de publier uniquement des séquences microsatellites sur les *Eucalyptus*.
Concrètement, une collaboration va débuter par l'envoi de 12 séquences microsatellites et 12 échantillons d'ADN à tous les participants de la table ronde (voir une partie de ces participants sur la photo 1). Le but de cet échange est d'intégrer ces échantillons dans l'étude de nos populations et d'utiliser les mêmes marqueurs. Il sera ainsi possible de relier nos études concernant la description de la variabilité génétique de nos différentes espèces et trouver un échantillon robuste de séquences transférables à ces différentes espèces. Ce travail pourrait être publié dans TAG.

Pour définir la collaboration internationale initiée lors de ce Symposium dont le but est d'augmenter nos connaissances du génome des *Eucalyptus*, le groupe a décidé de nommer cette collaboration sous le nom «Eucalypt Genome Initiative».

DISCUSSIONS SUR LES THÈMES DE RECHERCHE POUVANT ÊTRE DÉVELOPPÉS.

René Vaillancourt souhaite prendre une année sabatique et venir travailler dans notre laboratoire. Il viendra début juin pour visiter le laboratoire durant ses congés. René est canadien et occupe un poste d'enseignant chercheur à l'Université de Hobart en Tasmanie.

Chris Harwood propose des graines d'une provenance *E. pellita* de Papouasie Nouvelle Guinée dont les performances sont exceptionnelles. Il a également une très bonne provenance d'*E. urophylla* de Wetar

VISITE DE LA COMPAGNIE NORTH FOREST PRODUCTS.

L'activité principale de la North Limited est l'exploitation minière (or, argent, uranium etc ...), la compagnie exploite des mines et vends des minéraux. La compagnie North Forest Products est une filiale qui exploite la forêt et vend le bois. L'activité forestière est uniquement développée en Tasmanie. Depuis 1946 la compagnie gère 130 000 hectares de forêt naturelle avec des paysans qui sont associés par contrats. L'usine transforme le bois en chips et produit actuellement 2 millions de tonnes par an. La compagnie reboise les parcelles exploitées, avec des plants issus de semis. Les graines sont récoltées avec des nacelles (sans couper de branches).

Le programme d'amélioration génétique vise à augmenter la croissance, la qualité du bois et la tolérance au froid. La croissance est meilleure pour *E. globulus* et *E. nitens* a un meilleur rendement papetier. Après 10 années d'investissement dans la culture de tissus, les progrès ont été estimés trop faibles. Le programme a été arrêté, certains chercheurs ont été reconvertis (cas de Gillian Rasmussen qui est passée de la culture in vitro à la qualité technologique des bois).

ANNEXES

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Phylogeny of the eucalypts based on molecular and morphological data sets

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An overview of the phylogeny of the eucalypts, viz. *Angophora*, *Corymbia* and *Eucalyptus*, is presented based on published DNA sequence data (5S rDNA spacer), chloroplast DNA RFLP's and morphological characters. The molecular and morphological data sets proved useful at different levels in the eucalypt clade such that a resolved phylogenetic tree was obtained. The tree shows that *Angophora* and *Corymbia* (bloodwood eucalypts) are sister taxa, with *Eucalyptus* the sister group to them both. These findings support the recent taxonomic recognition of the bloodwoods as a separate genus.

Within *Eucalyptus*, three major clades, traditionally recognised as "eudesmids", "symphyomyrts" and and "monocalypts" are identifiable. The 5S molecular data, however, are insufficiently informative to resolve relationships of taxa within each of these three major groups. Other DNA regions are being investigated, together with morphological characters, for further resolution of phylogeny. Such phylogenetic analyses are the basis for improving the classification of eucalypts and for historical biogeographic studies.

Informativeness of nuclear and chloroplast DNA and relationships in the *Arillastrum* and eucalypt groups

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Phylogeny of the eucalypt group (sensu lato) is being studied using 6 regions of DNA: 5S rDNA repeat, ITS-1, ITS-2, *psbA-trnH* spacer, *trnL* intron, and *trnL* 3' exon-*trnF* spacer. Within the family Myrtaceae the genus *Lophostemon* was used as the outgroup to root trees and the ingroup included *Allosyncarpia*, *Arillastrum*, *Eucalyptopsis*, "Stockwellia", *Angophora* (3 species), *Corymbia* (3 species) and *Eucalyptus* (5 species). Levels of informativeness of each of the DNA regions is compared and phylogenetic analyses are presented for each data set and a combined (total evidence) data set.

Molecular phylogeny of the Myrtaceae and the root of the eucalypts

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The Myrtaceae is a family of over 130 genera that has traditionally been divided into 2 or 3 subfamilies based on fruit characters. Within these, a number of tribes and subtribes were recognised. These groupings have been reassessed cladistically using morphological and anatomical data by Briggs and Johnson (1979) and Johnson and Briggs (1984) who rejected the traditional subfamilial categories and proposed a series of "alliances" and "suballiances" in their place. To test these hypotheses, and to clarify suprageneric relationships in the family, we have sequenced the chloroplast gene *mat*K for around 100 taxa from all alliances plus the closely-related Psiloxylaceae and Heteropyxidaceae. Analyses of these data using *Sinapis*, *Saxifraga*, *Memecylon* and representatives of the Vochysiaceae as outgroups, have identified 10 robust clusters of taxa and a few genera in isolated positions. There is support for the *Acmena*, *Chamelaucium* and *Backhousia* alliances, and for the Myrtoideae sens. str. The *Metrosideros* and *Leptospermum* alliances are polyphyletic with part of the latter appearing as sister-group to the *Chamelaucium* alliance. The eucalypts and their allies form a strongly supported monophyletic group. Based on a sample of 8 taxa, we found three well-supported clades: *Corymbia* + *Angophora*, *Eucalyptopsis* "*Stockwellia*" and *Eucalyptus* sens. str. The position of the *Eucalyptopsis* group relative to the other clades and to *Arillastrum* is variable but it clusters with the latter and *Corymbia* + *Angophora* in the majority of trees. This topology is congruent with one of the two alternative trees of Hill and Johnson (1995).

Phylogenetic relationships among the eucalypts: evidence from the internal transcribed spacer (ITS)

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Sequence data for the internal transcribed spacer (ITS) regions of the nuclear ribosomal DNA were obtained from over 80 species of *Eucalyptus*, 15 species of *Corymbia*, 6 species of *Angophora* and one species each of *Allosyncarpia*, *Arillastrum* and *Stockwellia*. Multiple representatives of several species of *Eucalyptus* and *Corymbia* were also included in the analysis. A parsimony analysis of the full data set showed *Eucalyptus* to be monophyletic and well differentiated from the outgroup taxa. *Angophora* and *Corymbia* (the bloodwood eucalypts) formed a well-supported monophyletic group, with *Corymbia* forming two distinct clades. The data presented here support the recognition of the bloodwood eucalypts as a taxon separate from the rest of *Eucalyptus*. Subgenus *Eudesmia* does not form a monophyletic group; relationships among the six representatives included in the ITS analysis remained unresolved at the base of the *Eucalyptus s.s.* clade . Within the subgenus *Symphyomyrtus*, section *aidenaria* formed a well-supported monophyletic group; sections *Transversaria* and *Exsertaria* together formed a monophyletic group; and section *Bisectaria* appeared to be polyphyletic, comprising two distinct monophyletic groups. Subgenus *Telocalyptus* appeared to be embedded within subgenus *Symphyomyrtus*, suggesting that *Telocalyptus* may not deserve subgeneric status.

Evolution into the outback: molecular tests of taxonomy, phylogeny and vertebrate pollination in *Eucalyptus* series *Curviptera*

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Eucalyptus series *Curviptera* comprises some 25 species of mallees and small trees found mainly in Western Australia and adjacent deserts of South Australia and the Northern Territory. It includes several species with large colourful flowers of horticultural interest (e.g. *E. macrocarpa, E. youngiana*). In this study, we examined three hypotheses: (i) taxa defined by morphological and ecological characters are supported by molecular genetic variation patterns; (ii) relictual palaeoendemics are concentrated in the high rainfall forests of south-west Australia while derived neoendemics are found in the transitional rainfall wheatbelt and arid zone; (iii) vertebrate pollination is a derived system in eucalypts. Allozyme variation at 11 loci in 45 populations of 14 species of *E.* ser. *Curviptera* was investigated. While phylogenetic analysis of the molecular data supported continued recognition of several species, others had populations displaying significant divergence in allozyme frequencies (e.g. Pilbara populations of *E. kingsmillii* were widely separated from Gibson Desert populations). Basal taxa in the analysis included *E. virginia* ms, a rare palaeoendemic confined to the wettest forests of WA, and *E. lane-poolei*. Rare neoendemics such as *E. rhodantha, E. impensa,* and *E. rameliana* are from the transitional wheatbelt and deserts. The study documented a large cluster of taxa from the latter regions displaying low divergence and overlapping variation patterns, suggestive of recent evolution and/or extensive introgression. Predominantly vertebrate pollinated taxa were in this group, whereas species with generalist flowers were confined to wet country. The study thus supported the hypothesis that vertebrate pollination is a derived system in eucalypts.

CpDNA evidence for the role of hybridisation in *Eucalyptus*

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Four highly differentiated chloroplast DNA (cpDNA) lineages were identified in the forest tree species *Eucalyptus globulus* Labill. (Myrtaceae) using restriction site polymorphisms from Southern analysis. The cpDNA variation did not conform with subspecies boundaries, yet there was a strong geographic pattern to the distribution of the lineages. One lineage (C) was geographically central and widespread, whereas the other three lineages were found in peripheral populations (Western - W, Northern - N, and Southern - S). Thirteen haplotypes were detected in *E. globulus*, 7 of which belonged to lineage C. At least three of the cpDNA lineages (C, N and S) were shared extensively with other species (Steane et al. 1998).

On the east coast of Tasmania, there is a distinct north-south difference in cpDNA in the virtually continuous distribution of *E. globulus*. Northern populations harbour haplotypes from clade C while southeastern populations harbour a single haplotype from clade S. This difference is also reflected in several co-occurring endemic species. It is argued that the extensive cpDNA differentiation within *E. globulus* is likely to originate from interspecific hybridisation and "chloroplast capture" from different species in different parts of its range. Superficially this hybridisation is not evident in taxonomic traits, however large scale common garden experiments have revealed a steep cline in quantitative genetic variation which coincides with the haplotype transition in Tasmania. Our cpDNA results provide evidence that hybridisation has had a widespread impact on a eucalypt species and suggest that reticulate evolution may be occurring on an unappreciated scale in *Eucalyptus*.

Jackson HD, Steane DA, Potts BM, Vaillancourt RE (in press) Chloroplast DNA evidence for reticulate evolution in *Eucalyptus* (Myrtaceae). Mol Ecol

Steane DA, Byrne M, Vaillancourt RE, Potts BM (1998) Chloroplast DNA polymorphism signals complex interspecific interactions in *Eucalyptus* (Myrtaceae). Austr J Bot 11: 25-40

A useful hyper-variable region within the cpDNA genome of eucalypts

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Chloroplast DNA (cpDNA) provides useful markers for phylogenetic and population studies including gene flow and maternity studies. All the cpDNA studies in *Eucalyptus* to date have been based on the RFLP technique, which requires relatively large amounts of clean DNA and time. The development of cpDNA markers that avoid the RFLP technique would prove useful in studies that require DNA extraction from a large number of samples. This study aimed to develop PCR-based cpDNA markers and assess their usefulness in *Eucalyptus*.

The chloroplast genome of *Eucalyptus* possesses the inverted repeat (IR) characteristic of most angiosperms, such as in *Petunia* and *Nicotiana* (Byrne et al. 1993). There are two junctions between the large single copy (LSC) region and IRs, and these are defined as J_{LA} and J_{LB}. J_{LA} was sequenced from 21 DNA samples of *E. globulus* from the cpDNA RFLP study of Jackson et al. (in press) and J_{LB} was sequenced using five samples. These samples were chosen to represent all major haplotypes identified in that study. J_{LA} was 150 to 210 bp in size while J_{LB}. was approximately 500bp in size. Eighteen mutations were scored in total, most of which were found in the IR. Many of the characters were complex indels. The J_{LB}-LSC region had a low rate of base pair substitutions, with no informative mutations. The J_{LA}-LSC region contained 2 informative mutations. The variation uncovered from these sequences reflects the differentiation previously uncovered in an extensive RLFP analysis on the same samples (C and S haplotype). We concluded that sequencing the J_{LA} region would provide useful polymorphism for future studies.

We have subsequently sequenced J_{LA} from about 120 samples from all other *Symphyomyrtus* species in Tasmania (4 from series *Ovatae* and 12 from series *Viminales*) from throughout their geographical range. This data showed that: 1) the S and C haplotypes found in Tasmanian *E. globulus* transgress species and series boundaries; and 2) the geographical zone covered by the S haplotype of *E. globulus* is parallelled in other species.

Byrne M, Moran GF, Tibbits, WN (1993) Restriction map and maternal inheritance of chloroplast DNA in *Eucalyptus nitens*. J Hered 84: 218-220

Jackson HD, Steane DA, Potts BM, Vaillancourt RE (in press) Chloroplast DNA evidence for reticulate evolution in *Eucalyptus* (Myrtaceae). Mol Ecol

The role of molecular markers in the conservation of eucalypts

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Molecular markers have helped to advance the conservation of eucalypts in several ways. Allozyme studies provided important insights into species limits, population genetic architecture and mating systems, facilitating design of reserve systems and seed orchards, as well as management and restoration of populations. Continuously distributed forest eucalypts display high levels of variation within populations, and low geographic variation, whereas eucalypts with disjunct population systems have higher between-population variation. Small population size is often associated with loss of genetic variation and manifest inbreeding effects, except where clonality and bird pollination play compensatory roles. Use of DNA markers has enabled more penetrating analysis than is possible with allozymes, elucidating questions of hybridity, clonality and phylogeny. Some of these issues will be highlighted by recent case studies from Western Australia involving extremely rare mallee eucalypts such as *E. x graniticola, E. phylacis,* and *E. dolorosa.*

Integrating quantitative and molecular genetic approaches to study forest sub-structure

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The generally limited seed dispersal in *Eucalyptus* has caused many authors to hypothesise that native forest may be a mosaic of family groups, consisting of individuals sharing at least a half-sib relationship. Such fine-scale genetic sub-structuring may have a marked affect on the levels of inbreeding and has important implications for sampling and assessing genetic diversity in natural forests. Molecular and quantitative genetic approaches were used to study the spatial pattern of relatedness in native forest of *Eucalyptus globulus* ssp. *globulus*.

The association between geographic distance and genetic similarity based on 69 RAPD markers was studied among 51 trees from the Tinderbox locality in southern Tasmania (distance ranging from 2 m to 4 km apart) and compared to 18 trees from localities up to 100 km away. Twenty pedigreed F_1's were used as controls to scale the RAPD similarity among individuals to pedigree similarity. The association between genetic similarity and geographic distance was weak, yet at Tinderbox, highly related trees were shown to occur within 25 m of one another. There is an abrupt drop in average similarity after about 25 m, with no significant change with distances up to 14 km. Nevertheless, Tinderbox trees outside the 25 m genetic patches are still more similar to each other than they are to trees from the Mayfield Bay locality 100 km away. The molecular results suggest that *E. globulus* native forests have a family group structure, superimposed on a noisy, background level of lower relatedness which extends over a wider geographical range. This conclusion was consistent with results obtained from crossing a subset of the trees used in the molecular study. Inbreeding depression is severe in *E. globulus*, and reduced vigour of progenies from crosses amongst proximate parents was used as an indirect measure of parental relatedness. Seven trees were used as females and crossed with pollen from trees at increasing distance: 0m (selfing); 21m (nearest flowering neighbours), 250m, 500m, 1km, 10km, and 100km away from the females. Growth of the 21m progenies was intermediate to selfing and the longer distance crosses suggesting nearest-neighbours are related. However, there was little change in progeny vigour when parents were separated by 250m or greater distances, consistent with the results from the molecular study.

This study is unique in revealing similar fine-scale genetic structure using both molecular and quantitative genetic approaches. Under this population structure, biparental inbreeding between proximate relatives may be common. However, it is argued that inbreeding would not normally accumulate due to intense selection against the products of inbreeding, preventing a build-up of homozygosity. Selection would favour the products of longer distance pollinations and the spatial pattern of relatedness re-established each generation due to limited seed dispersal.

Intraspecific phylogeography and genetic structure in eucalypt species

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Structuring of genetic variation within and between populations is related to current patterns of gene exchange and historical relationships (Schaal *et al.* 1998). Studies of genetic variation have generally been concerned with the variation in the nuclear genome, which provides information on current patterns of genetic diversity which are strongly influenced by gene exchange. Historical events leading to patterns of common ancestry will have strong effects on population genetic structure, and are often not distinguished from recurrent processes determined through assessment of the nuclear genome. However, variation in the chloroplast genome can complement nuclear diversity since gene genealogies provide a historical approach to the study of intraspecific processes (Schaal *et al.* 1998).

Intraspecific phylogeography of two eucalypt species with contrasting distributions will be discussed. The *E. kochii/E. horistes* group has a localised distribution in Western Australia, and shows high genetic diversity with little population differentiation. Congruence between chloroplast gene genealogies and geography, with restricted distribution of derived haplotypes, indicates restricted gene flow and a degree of population isolation. Patterns of population phylogeny showed congruence. *E. nitens* has a disjunct but widespread distribution in eastern Australia , and shows high genetic diversity with strong regional differentiation. Incongruence between geography and gene genealogy suggests the presence of two lineages with differential linage sorting occurring within populations.

Schaal, B.A., Hayworth, D.A., Olsen, K.M., Rauscher, J.T., and Smith, W.A. (1998). Phylogeographic studies in plants: problems and prospects. *Molecular Ecology* 7, 465-474.

Impacts of reforestation practices on genetic diversity of *Eucalyptus sieberi*

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We are employing DNA markers to examine the effects of silvicultural practices on genetic diversity in the Australian native forest species *Eucalyptus sieberi* L. Johnson. The field site for this experiment is provided by the Silvicultural Systems Project of the Victoria Department of Natural Resources and Environment, near Orbost, Victoria. Genetic impacts of three different silvicultural treatments: 1)_clear-felling with aerial re-sowing; 2) the seed tree system with site preparation by burning; and 3) the seed tree system with site preparation by mechanical disturbance are being assessed with the markers. The entire data set being collected will consist of 30 RFLPs and 10 microsatellites assayed on a total of 825 trees. Results so far, from two of the silvicultural treatments (clear-felling and the seed tree system with site preparation by burning), reveal a high level of genetic diversity in this locally common species, and little or no loss of diversity due to either of these silvicultural treatments. However, cluster analysis via UPGMA detected some genetic drift due to the seed tree regeneration method. The most recent results from this project will be presented and discussed.

Genetic variability analysis of a base-population of *Eucalyptus* using RAPD markers

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The level of the variability within forest species so far has been assayed using morphological markers, which are influenced by the environment and show little polymorphism. Molecular markers can change this situation since they allow a more precise analysis and can generate a large amount of data in a short time. This study aimed to analyse the genetic variability and genetic distances within base populations of *E. urophylla* and *E. grandis* to guide breeders in taking actions to increase their level of genetic variability and build a molecular data bank of these populations. The population of *E. urophylla* was composed of 61 individuals from Flores (Mt. Egon), Timor and other islands, all selected from different progeny tests and in commercial plantings. The population of *E. grandis* was composed of 327 individuals mainly from Coffs Harbour and Artherton. RAPDs were used to analyse the genetic variability in each population. The RAPD technique allowed the analysis of 70 loci and genetic distance matrices were generated using Jaccard's Coefficient. The average genetic distance among the individuals analyzed of *E. urophylla* was 0.77 and among the individuals of *E. grandis* was 0.67. RAPDs allow the assay of the genetic variability in the populations analyzed and they allow the choosing of the most genetically different plants for hybrid production. Our data showed many redundancies in the sample analyzed and they indicate the genetc basis of some populations should be increased. A higher level of polymorphism was detected in experimental planting of *E. grandis* than in a population from Australia, after a single generation of recombination.

The use of SSR markers to study gene flow in South American *Eucalyptus* seed orchards

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Since many *Eucalyptus* species are now widely grown for timber or pulp outside their natural range it is not clear how efficient pollination will be in the absence of naturally adapted pollinator species. We have developed a range of SSR markers from an (CA)n enriched genomic library in order to investigate the patterns of gene flow in a *Eucalyptus globulus* seed orchard in Chile and a *Eucalyptus grandis* seed orchard in Uruguay. The SSRs from our library show very high levels of heterozygosity which make them ideal for tracking pollen mediated gene flow under these conditions. In addition, we have found that primer pairs designed for our *Eucalyptus globulus* sequences are capable of amplifying polymorphic microsatellites not only from *Eucalyptus grandis* but also from a wide range of economically important *Eucalyptus* species. This suggests that such SSR markers will have a wide range of applications in *Eucalyptus* forestry and genetics ranging from the fingerprinting of clonal lines to QTL analysis.

Currently we have completed the experimental component of our *Eucalyptus grandis* study. Preliminary results indicate that pollination within the orchard is highly efficient with a high number of trees contributing as pollen parents to the seed output from a single maternal parent. In addition, there is relatively little evidence of pollen mediated gene flow into the seed orchard from surrounding plantations.

Beyond QTL mapping: challenges to incorporate QTL information into eucalypt breeding

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The major expectation of molecular breeding in forest trees is that markers will allow tracking the introgression of favourable alleles in early selection procedures. The challenge for the application of Marker Assisted Selection in forest trees is however more complex than in crop plants as it presupposes: (1) the manipulation of polygenic traits with variable heritabilities in genetically heterogeneous populations; (2) its incorporation in breeding schemes that involve altering frequencies of favourable alleles through recurrent selection and intermating; (3) dealing with age x age trait correlations and variable environments. All QTL mapping studies in *Eucalyptus* have successfully demonstrated experimental approaches for locating QTLs and provided evidence for the numbers and effects of QTLs controlling quantitative traits such as volume growth and frost tolerance. However the use of the linkage information tends to stay restricted to the pedigree employed as the mapping population, limiting the inter-experimental sharing of QTL data. Furthermore, due to the small progeny sizes and number of genetic backgrounds, limited precision in phenotype assessment and information content of the molecular markers used, the true numbers, precise position and magnitude of effect of QTL alleles detected is still insufficiently understood. Results from a limited number of studies point to the fact that in tropical *Eucalyptus* QTL x age interaction might not be a major limitation for the implementation of early MAS. However a particular QTL allele detected in a particular pedigree might not be the top allele existing in the breeding population and other alleles of equal or even better effect may exist. The success of MAS will therefore depend heavily on the ability to discover, map and rank the effect of the alleles that exist at major QTLs in a breeding population. The challenge is not QTL mapping but rather understanding the allelic diversity at known QTLs. To carry out such ranking, a reference linkage map of transportable multi allelic microsatellite markers will be a must. Only by altering the frequencies of QTL alleles of large effects will MAS surpass conventional phenotypic selection and be useful for breeding. In the context of *Eucalyptus* breeding, the prospects are positive. Small breeding population for hybrid performance combined with clonal propagation are favourable conditions for MAS. Top alleles of trees in specialised breeding populations could be tagged and ranked by analysing the performance of offsprings in factorial mating designs. MAS would be most likely applied for the improvement of multiple elite populations developed specifically for extreme quality trait values such as wood properties and stress tolerance. This effort should target the most valuable individuals in a breeding population, and probably begin by working with only 1 or a few traits in a specific breeding group. The effective incorporation of MAS in *Eucalyptus* breeding still needs to be demonstrated and will require further experimental work. Today, complete genome sequences and new enabling technologies based on DNA chips are opening new avenues for screening single nucleotide polymorphisms and functional variation. These technologies should quickly become routine and potentially can revolutionize the way we approach molecular breeding of forest trees.

Syntenic mapping and QTL characterisation in eucalypts

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There is a growing body of evidence of high levels of synteny within biological groups such as grasses and mammals. This map information coupled with gene sequences from model plant species like *Arabidopsis* and rice will be a crucial resource for the advancement of molecular breeding of forest tree species. In eucalypts, linkage maps have been constructed in a limited number of species and only a few of these maps have been comparable through the same markers. The species have been commercial species belonging to three evolutionary groups in the subgenus *Symphyomyrtus* with the exception of *E. marginata*, a monocalypt species. Moreover, in only a few species have multiple pedigrees maps been used to make maps. The ultimate aim will be to have a robust reliable generic framework map and CSIRO already has a first pass at such a map. It obviously requires a set of suitable markers that can be used across species. The markers in order of preference are genes (including isozymes), RFLPs and microsatellites. RFLPs work across the genus but have the limitation of not being PCR-based. Microsatellites work well across species within a subgenus but not across subgenera. Homologous genes from model plant species and candidate genes from known biosynthetic pathways for commercial traits will increasingly be the focus of mapping and will enable testing of synteny from eucalypts to other plant families.

Estimates of synteny will ultimately depend on the ability to construct correct maps. Not only will markers need to be orthologous but the ordering of markers in linkage groups for outcrossing species needs to be improved. One of the limitations has been sample sizes of pedigrees. Another limitation has been statistical methods employed in linkage programs to order markers but research underway should lead to better methods. Hopefully this will result in increased power to detect rearrangements within linkage groups and lead to estimates of the commonality of such phenomena within breeding populations and across species. In eucalypts a number of QTL have been detected for a number of traits but concern is evident about accuracy of detection and size of QTL effects given sample sizes available. The extent of validation of QTL is a current major research topic as is methods to extend this information to breeding populations. These are obviously also concerns for commercial forest tree species generally. The application of QTL data in molecular breeding may ultimately depend on the nature and origin of the breeding populations of particular forest tree species.

Linkage map of an intra-provenance cross of *Eucalyptus globulus* using RAPD and microsatellite markers

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In recent years there has been several DNA based marker maps published for individuals in the genus *Eucalyptus*. These maps have been constructed from inter-specific crosses (*E. grandis* x *E. urophylla, E. globulus* x *E. tereticornis*) or from an inter-population cross (*E. nitens*). Unfortunately it has not been possible to identify homology between linkage groups (synteny) in the different published maps. Also to date there have not been any maps published for *Eucalyptus* that have been derived from within population crosses.

In this study an intra-provenance cross of *Eucalyptus globulus* with 165 F_1 progeny was used for mapping. This family is part of the CSIRO/North Forest Products hybrid trials. Two marker maps were constructed, one for each of the two King Island derived parents. The maps are composed largely of RAPD markers with a number of interspersed microsatellite loci. The mapping program MAPMAKER was used for linkage analysis and marker ordering. For the male parent, 180 RAPD markers, 15 microsatellite markers and one isozyme locus were used to construct the linkage map. When loci were grouped using the threshold values of min. LOD 5.0 and max. recombination fraction (θ) of 0.3, 13 linkage groups were obtained (1290 cM). For the female parent, 150 RAPD loci and 14 microsatellites were used for map construction. At LOD 5.0, $\theta = 0.25$ MAPMAKER assigned loci to 12 linkage groups (700 cM). More segregation distortion than expected was found in the female parent but not in the male parent. Synteny was determined for 8 linkage groups of the two parents, as indicated by the sharing of 11 microsatellite loci.

We demonstrate that the RAPD assay can readily provide sufficient polymorphic markers to produce maps from an intra-provenance cross, a cross type that is expected to have little diversity. The mapping of eighteen microsatellite loci indicates the utility of these markers for mapping and allowed homology between most linkage groups of the two parents of the cross to be determined. The broad transferability of microsatellites is also indicated since the microsatellite primer sequences were derived from 5 species of *Eucalyptus*. Some comparison with the *E. grandis* and *E. urophylla* maps of Brondani *et al.* is possible since 7 of their microsatellite loci have been mapped establishing the potential for future map integration, transference of map positions and sharing of other map based information. The maps generated in this study will now form the basis for a search for QTL (Quantitative Trait Loci) for wood density (pilodyn penetration) and growth (d.b.h.).

QTL detection and genes mapping for clonal selection in a *Eucalyptus* breeding program

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The objective of this study was to present the main researches used with molecular markers within the context of the reciprocal recurrent selection scheme developed by the CIRAD-Forêt on *Eucalyptus* breeding program for the tropical region of Congo.

Saturated genetic maps of two parental elite trees have been described elsewhere. Those comprise 269 and 236 RAPD markers for the *Eucalyptus urophylla* and *Eucalyptus grandis* individual parents, respectively. This interspecific hybrid family was used to determine the genetic location and effects of genomic regions controlling wood quality, stem growth and form and rooting of cuttings in the *Eucalyptus grandis* and *Eucalyptus urophylla*. The progenies were studied from the plantation in 1992 to the exploitation in 1998. Several regions controlling part of the traits variation were identified by interval mapping and ANOVA. After 3 years some markers were significantly associated with vigour and form and the chromosomal regions were stable over the studied period.

In order to estimate the co-localisation between gene and QTL, we have started another study in genomic science. The objective is to obtain information about the variability of lignin contents. The localisation of lignification genes was started with single strand conformation polymorphism (SSCP) method on our two single-tree linkage maps. Five genes with emphasis on lignification: PAL, COMT, CCoAOMT, CCR, and CAD were mapped on our previous genetic linkage groups. Four other genes (C4H, 4CL, C3H, F5H) will be studied in another project. In addition, two genes affecting cellular division and rooting ability: EgTub and EgPar have been mapped.

The association between RAPD polymorphism and the interspecific additive mean was evaluated in a factorial plan, for wood density at 18 months and vigour at 38 months. Now we are looking for the possible use of such information, firstly in order to select the parents for further generations of breeding, and secondly in order to choose the hybrid families in which QTAs of specific value could be detected and used to identify and select the best trees These results are very encouraging for the application of marker information towards early selection of hybrid trees to be vegetatively propagated for clonal production.

Genetic mapping of monoterpene composition in an interspecific eucalypt hybrid

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Genetic control of foliar oil composition was investigated amongst half-sib progeny of an interspecific eucalypt hybrid. The oil was found to be largely composed of the monoterpenes, limonene, a-pinene, g-terpinene, 1,8 cineole and *p*-cymene. Due to difficulties in the interpretation of compositional data based on raw proportions, further analysis was conducted using logratio variables. A high degree of intercorrelation amongst logratios was thought to be a consequence of commonality in the biosynthetic origins of the monoterpenes. Quantitative trait loci (QTL) analysis of logratio variables indicated that a significant (68-81%) proportion of the variation in four out of the ten possible logratios were controlled by a single genomic region of the maternal *Eucalyptus grandis* parent. The impact of this genomic region upon oil composition was thought to be a consequence of a gene or genes controlling the production of limonene as limonene was the predominant oil constituent in many hybrid individuals and was common to all logratios associated with the identified genomic region.

Gene action of multiple alleles that control *in vitro* rooting ability of *Eucalyptus nitens* detected with codominant RFLP and microsatellite markers

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Vegetative propagation of superior trees of *Eucalyptus nitens* by tissue culture has the potential to increase operational gain per unit of time from genetic improvement programs. Ability to set roots *in vitro* would enable rapid, large-scale deployment of newly developed genetic materials in plantations. However, only a small percentage of desired *E. nitens* trees can be successfully cloned at present. The detection and localisation of quantitative trait loci (QTLs) will lead to a greater understanding of the genetic control of rooting. This will facilitate the development of efficient strategies for selection of clones with high rooting ability.
Identification of QTLs in outbreeding pedigrees has shown the presence of multiple alleles and evidence of intra- and inter-locus interaction. Full-sib families can show segregation of up to four alleles at both molecular and quantitative trait loci (QTL). In such cases, the use of fully informative codominant markers to tag QTLs allows inferences to be made on the mode of action of the QTLs.

We are using a framework genetic linkage map of *E. nitens* developed with codominant RFLP and microsatellite (SSR) markers to map regions of the genome controlling ability to set roots *in vitro*. Two full-sib families of *E. nitens* with one common parent have been selected, one family (N = 327) will be used as the mapping pedigree and the other (N = 207) for verification of putative QTLs. Results obtained showed low repeatability of percent rooting *in vitro* over four temporal assays. Results from QTL analysis will be presented.

A molecular approach towards wood quality improvement in *Eucalyptus*

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The performance of wood and wood products for various end uses, which can be as divergent as pulp and paper and solid wood, is largely dependent on biological processes underlying the differentiation of a vascular cambium and the subsequent formation of woody tissues. A major proportion of this biological basis of wood formation is under genetic control. To better understand the molecular basis of wood formation we have used three approaches to isolate genes which are prominently involved in this process: (i) the use of heterologous probes for screening of an *E. globulus* cambial cDNA library; (ii) the use of specific and degenerate PCR-primers for amplification of corresponding eucalypt sequences and (iii) the isolation of tissue specific cambial sequences using a subtractive approach with subsequent suppression PCR. These approaches have yielded a number of novel cambially expressed eucalypt cDNA fragments as well as fragments of many known genes that are involved in meristematic activity and cell proliferation, cell (fibre) morphology, lignin composition and content, cellulose biosynthesis, other cell wall features, hormone biosynthesis, and a number of other traits. For some of those, strong cambium-specific expression could be established. While many of these genes can now be used as target genes for the genetic manipulation of wood quality parameters, the cambium-specific promoters enable us to do so in a strictly localised way.

Population diversity in *Cryphonectria cubensis* and its implication for *Eucalyptus* breeding

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Cryphonectria cubensis is one of the most destructive pathogens of *Eucalyptus*. The disease is particularly severe in tropical and sub-tropical countries where species such as *E. grandis* and hybrids of this and other species are extensively propagated. Reduction of losses due to *C. cubensis* is most effectively achieved through breeding and selection of disease tolerant clones. The durability of this tolerance is inextricably linked to the genetic diversity of the pathogen. We have studied populations of *C. cubensis* from various South American countries, from Indonesia and South Africa. These studies have been based on various techniques such as ribosomal DNA sequencing and the vegetative compatibility between hierarchical collections of isolates of the pathogen. Our studies have confirmed the fact that the clove canker pathogen, *Endothia eugenia,* is the same as *C. cubensis.* We have also shown that sexual reproduction is common in the fungus in Indonesia and various South American countries but that it appears to be absent in South Africa. Furthermore, *C. cubensis* has a very limited genetic diversity in South Africa which is indicative of a recently introduced pathogen, and the absence of sexual recombination. In contrast, *C. cubensis* populations from all other countries considered appears to be represented by a very diverse population, which indicates that it is either native or has been present for an extended period of time as a sexually outcrossing population. The low level genetic diversity *of C. cubensis* in South Africa suggests that tolerance to *Cryphonectria* in this country will be more durable than it is likely to be in Indonesia and South America.

Star Tree: the next generation

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With production of genetic maps now a routine procedure, our focus is on how to best use the abundance of marker information generated to more effectively detect QTL. One improvement is better analysis procedures to accommodate limited family sizes and rogue data. The new multiple interval mapping (MIM) system has proved to be very powerful, providing better estimates of position and size of QTL, as well as accommodating linked QTL and epistatic interactions. The substantial time demand of this analysis has now been dramatically eased with improved software, resulting in considerable increase in the number of QTL reliably identified. Traits for which QTL have been identified include height, diameter, density, branch character, flowering, insect resistance, and terpene composition. Marker bred hybrid eucalypt trees (various generations of *E. urophylla* x *E. grandis*) have been cloned and monitored in field trials, with some individuals exhibiting heights over 13 m at 18 months. Field performance data are now allowing progressive selection of elite clones. This general strategy is being applied to a variety of acacia, pine and eucalypt species, including introgression of salt tolerance from *E. camaldulensis* into elite *E. globulus* and *E. grandis* germplasm. Clonal propagation systems have been developed which are generally genotype-independent to allow full capture of genetic potential. Robotic systems have also been developed for high volume micropropagation. We now have over 2000 different clones at various stages of commercial development.

Introduction of exotic genes is generally only appropriate when target traits are not available in the natural gene pool. We have a variety of useful genes available to us, both from our own studies and under licence, including salt and stress tolerance, root-induction, insect resistance, and herbicide resistance. A gene-discovery program has been in progress for some time with *ca.* 15,000 cDNAs sequenced. Transformation/regeneration systems have now been developed for five species of eucalypts, as well as *Pinus radiata*. In order to deploy transformed trees, sterility has been engineered using both cell-ablation and anti-sense strategies. This has involved characterisation of a suite of both our own and licensed genes involved in early stages of floral development. In parallel with sterility we have also engineered early flowering, to allow shortening of the generation interval for accelerated breeding. Work continues to extend the range of species to which these methods are applied, with integrated use of quality germplasm, marker-guided breeding and selection, transformation, and efficient clonal propagation, to permit capture of compounded genetic improvements.

Development of a genus wide reference linkage map for *Eucalyptus* based on microsatellite markers

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Similarly to human genetics in the pre-microsatellite era, a key obstacle to more precise detection of QTL and marker assisted selection in forest trees is the outbred nature of the pedigrees available and the limited information content of the molecular markers mapped to date (e.g. RAPD and AFLP). Microsatellite maps will represent a dramatic improvement in our ability to carry out high quality mapping experiments and implement marker assisted selection. We are constructing a genus wide reference map using a segregating population of 96 F_1 individuals from an interspecific cross of *E. grandis* X *E. urophylla*. Until December 1998, 120 EMBRA (*Eucalyptus* Microsatellite from Brazil) loci were typed in the mapping population. Out of these, segregation data of 81 SSR were analyzed. At LOD 5, 71 (89%) loci were placed on the *E. grandis* map in 13 linkage groups, and 48 (60%) in the *E. urophylla* map in 11 groups. Forty-three (54%) markers were fully informative, segregating in both parents, of these 39 were mapped allowing the establishment of synteny for all linkage groups between the two maps. A first set of 30 selected anchor loci were characterized for allelic content and expected heterozygosity in *E. grandis* and *E. urophylla* using a panel of 32 individuals. Given the very broad genetic base of *Eucalyptus* breeding programs, and expected heterozygosities > 0.75 for all loci, we anticipate that essentially every family segregating for traits of interest should provide marker-QTL linkage information. Transportability of SSR loci among pedigrees not only within the same species but also across species of the same subgenus should allow the determination of QTL synteny and facilitate a directed search for new allelic variation at known QTL within and among species. Most QTL in *Eucalyptus* and in other genetically heterogeneous forest trees are likely to be multiallelic, only multiallelic markers will permit tracking, understanding and adequately manipulating allelic variation at QTL. Our objective is to map a total of 150 EMBRA loci in the *Eucalyptus* reference map by Jan. 1999 and >250 loci by the end of 1999 with a set of fully characterized 80-100 anchor loci combined in 20-30 multiplexed systems to allow high throughput genome scan. The prospect of a reference map useful for the great majority of commercially important eucalypt species is now real. It will allow rapid spreading of these markers in *Eucalyptus* genetics research, facilitating information exchange and collaborative projects. Transportability of SSR loci across species will allow the determination of interspecific QTL synteny and facilitate a directed search for new allelic variation at known QTL expanding the opportunities for marker assisted introgression and selection in hybrid breeding.

The down-regulation of a-tubulin expression during the development of salt tolerance in *Eucalyptus microcorys*

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Salinity of soils is an increasing problem throughout many areas of Australia and the world, and has been recognised as being one of the most important environmental problems which can limit plant growth. Salt treatment elicits a number of responses in plants ranging from inhibition of growth and photosynthesis, to readjustment of metabolic processes and compensation for osmotic and ionic changes. It has also been shown that salt and water stress is controlled by an array of genes with many different functions; that is salt tolerance is controlled by complex multigenic traits.

A salt sensitive woody species, *Eucalyptus microcorys* was used to investigate the molecular effects of NaCl during the salt adaptation process in vitro. Non-salt exposed shoots and salt conditioned shoots exposed to 100 mmol/L NaCl, were examined using a technique known as differential display-reverse transcriptase PCR (DDRT-PCR). This method is particularly powerful for the study and determination of genes regulated at the transcriptional level. The technique is based upon the isolation of sub-populations of undegraded mRNA. A sub-set of the mRNA is utilised as template for representative cDNA synthesis by reverse transcription using a oligo dT primer in order to produce ssDNA. PCR is then performed on these cDNA sub-populations using an anchored and a random decamer primer. The use of these combinations of primers will generate a series of comparative cDNAs that corresponds to a sub-fraction of the mRNA present in the extract, and differences between samples detected.

Using DDRT-PCR, we found that there were several polymorphic changes between the two cultures (ie. salt sensitive and salt conditioned). One very strong band that was present in the non salt exposed shoots, but not in the salt conditioned shoots was cloned and sequenced; which was 84 % analogous in base sequence to α-tubulin. α-tubulin has a critical role to play in cell division and maintaining cell structure and turgidity, and therefore was consistent with the structural and physiological differences already observed in salt sensitive and salt conditioned *E. microcorys*.

Allozyme variation in the rare and endangered Tasmanian endemic *Eucalyptus morrisbyi*

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Eucalyptus morrisbyi R.G. Brett (Morrisby's Gum), endemic to Tasmania and now only found in a few locations near Hobart, is one of the rarest and most endangered of eucalypts. The data to be presented were collected in 1989 from two populations, Calverts Hill and Risdon Hill, separated by about 20 kms and differing greatly in the number of *Eucalyptus morrisbyi* trees present. The Calverts Hill population is the largest for the species and comprises about 2,000 trees over 11.5 hectares. The outlying population at Risdon Hill, in the East Risdon Nature Reserve, had 16 mature trees in 1989 but many have since died and the population now comprises mainly immature coppice regeneration.

Considerable allozyme variation was found in both populations, with differences between the populations in the alleles present and the allele frequencies. In particular, for one malate dehydrogenase locus, a number of the trees at Risdon Hill were heterozygous for an allele not present in the Calverts Hill population. This allele was also found in the nearest *Eucalyptus urnigera* (from Mount Wellington) but not in the nearest *E. gunnii* (from Snug Plains), which are Tasmanian endemics closely related, but allopatric, to *E. morrisbyi*. The allele was not found in the sympatric *E. viminalis* from the Risdon Hill locality, consistent with the absence of morphological evidence for hybridisation between *E. morrisbyi and E. viminalis* (Wiltshire *et al*, 1991) despite their close spatial proximity, overlapping flowering season and ease of artificial hybridisation.

Compared to other eucalypts, *Eucalyptus morrisbyi* has a high frequency of individuals incapable of setting seed following self-pollination, although only the Calverts Hill population has been tested. Seedlings from individual trees in each population were assayed and estimates of the level of outcrossing for the two populations and for individual trees will be presented.

Wiltshire, R.J.E., Potts, B.M., and Reid, J.B. 1991. Phenetic affinities, variability and conservation status of a rare Tasmanian endemic, *Eucalyptus morrisbyi* R.G. Brett. *In* Banks, M.R., *et al.* (Eds). *Aspects of Tasmanian Botany - A Tribute to Winifred Curtis.* Roy. Soc. Tasm. Hobart: 213-229.

Development of high throughput genotyping systems for *Eucalyptus* based on fluorescence detection of microsatellites

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We describe the development of semi-automated multilocus genotyping systems for *Eucalyptus* species based on fluorescent detection of multiplexed microsatellites. These systems allow large scale genotyping of natural and breeding populations of *Eucalyptus*, for individual discrimination, parentage studies, clone protection and molecular breeding. From a screening of 21 EMBRA (*Eucalyptus* Microsatellites form Brazil) loci, developed from *E. grandis* and *E. urophylla*, a set of loci was selected based on robustness, allelic content and transferability among *Eucalyptus* species. Three multiplex systems each with three fluorescently labelled co-amplified loci were developed for analysis on a ABI-Prism 377. Two systems that generated distinct size range products could be multiplexed at gel loading yielding up to 6 amplified loci in a single lane. Detailed characterization of these multiplex systems for genetic informativeness was carried out in *E. grandis* by genotyping 240 individuals from five distinct provenances. The large number of alleles and their homogeneous frequency distribution resulted in single-locus exclusion probability and polymorphism information content ranging from 0.53 to 0.87 and 0.74 to 0.93 respectively. The probability of identity for each multiplex system ranged from 5.4×10^{-4} to 3.6×10^{-6}. For all nine loci combined it reached $1,6 \times 10^{-14}$, which is the probability that two individual trees will have the same multilocus genotype. These results clearly illustrate the extraordinary power of discrimination of this genotyping system for *Eucalyptus*. These same nine loci were also characterized for other five important commercial species of the genus: *E. saligna, E. globulus, E. dunnii, E. urophylla* and *E. camaldulensis*. A total of 60 individuals per species sampled from 2 to 5 provenances were used, so that allele frequency estimates were obtained on 120 chromosomes per species. Although absolute transferability and high information content were seen for all 3 multiplexes, significant differences in the distribution of allelic frequencies were seen among species, particularly so for *E. urophylla*. This genotyping system provides a very powerful reference tool to carry out population genetics studies, clone fingerprinting and across lab/country comparative genetic analysis for some of the most planted species of *Eucalyptus* in the world.

Fingerprinting *Eucalyptus* genotypes with highly informative microsatellite markers

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Microsatellites are becoming increasingly popular and accessible for population genetics studies, linkage mapping and QTL analysis in plants. A powerful application of these markers is the generation of individual specific multilocus fingerprints to discriminate closely related individuals in parentage studies in breeding and natural populations. Here we report the DNA typing of 192 selected elite genotypes derived from 18 provenances of *Eucalyptus grandis* with a set of six highly polymorphic microsatellites (EMBRA - Eucalyptus Microsatellites from Brazil). Number of alleles detected ranged from 6 to 33 with an average of 19.8"9.2. The highest expected heterozygosity (He = 0.94) and polymorphism information content (PIC = 0.93) were observed at locus EMBRA-7, while among all the microsatellite loci the average was 0.86"0.11 and 0.83"0.16, respectively. With only 3 loci all the 192 genotypes could be readily discriminated. The combined probability of identity (L) considering all 6 EMBRA loci was less than 1 in 2 billions, demonstrating the higher resolving power of SSR-based markers in *Eucalyptus*. To evaluate the minimum number of individuals required to obtain accurate estimates of genetic informativeness indices (He, PIC and L), 1000 bootstrap samples of sizes n=24, 32, 64 and 128 were taken from the population. With the new allele frequency distributions obtained for each sample size the accuracy was estimated for each index estimating the coefficient of variation (CV_{ems}). No significant decrease in CV_{ems} was seen with n>64, indicating that 64 individuals should be an adequate sample size to be genotyped for estimating these parameters for SSR markers in *Eucalyptus*.

Genetic variability analysis of a base-population of *Eucalyptus urophylla* S.T. Blake using RAPD markers

Susi Meire Maximino Leite[1] Cesar Augusto Bonine[2], Celina Ferraz Valle[2] and **Celso Luis Marino**[1]

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This study aimed to analyze the genetic variability and genetic distances within a base population of *E. urophylla*, to help in the increasing of the genetic variability and to build a molecular data bank of this population. This population is composed by 61 individuals from Flores Mt. Egon, Timor and other islands, all selected among different progeny tests and in commercial fields. The individuals of the population were divided into 6 sub-population according their origins. RAPDs were used to analyze the genetic variability in whole population and in each of the 6 sub-populations. Seventy loci were analyzed, being 0.554451 the average genetic distance between the individuals analyzed. The progeny tests AE140 and 141 showed the highest level of genetic variability among the sub-populations, with an average genetic distance value of 0.648854. The progeny tests AE 147 and 148 showed the lowest genetic variability, with an average genetic distance of 0.544835.

RAPD analysis of the genetic variability within a breeding population of *Eucalyptus grandis* Hill.

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The level of the variability within florets species so far has been assayed using morphological markers, which are influenced by the environment and show little polymorphism. The molecular markers can change this situation since they allow a more precise analysis and can generate a large amount of data in a short while. We aimed to study the variability and genetic distance within a base population of *Eucalyptus grandis*, to help the improvement of the genetic bases and to build a data bank of molecular markers from the populations analyzed. The base population of *Eucalyptus grandis* analyzed is composed by 327 individuals mainly from Coff's harbour, Artherton; few plants were from some other locations (Belthorpe Mt. Pandanus, Kenilworth, Yabbra, Rifle Range, Mt. Lewis, Paluma, Mt. Spec, Mt. Frazer, etc). Because the heterogeneous nature of this population, the base population could be divided into groups according the latitude and longitude, and into sub-populations according the level of genetic breeding, what allowed to assay how much of the genetic variability detected was due to those factors. The RAPD technique allowed the analysis of 70 loci which were analyzed using Jaccard's Coefficient, what resulted in a genetic matrix. The data showed the base-population has a wide genetic bases, with a mean genetic distance of 0.672091. The sub-population sub-group 3 (wild material form the macro regions of Atherton), which is composed by 68 individuals and had a mean genetic distance of 0.682343, and the samples of local races from the macro-region of Coff's Harbour, which showed a mean genetic distance of 0.678503 contributed with the largest amount of variation to the whole population. This was due to the fact this sub-populations had the largest number of individuals in the base-populations (48.3% of the total number of individuals of the base population). Plants in the base population with genetic distance lower than 0.40 may be submitted to a new phenotypic analysis to decide more clearly on the heading of the genetic bases during breeding programs.

Determination of the origin of clones of *Eucalyptus* using RAPDs

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The molecular characterization of plant germplasm can yield data that can help plant breeders in breeding programs, and they allow the genotypic evaluation in very early stages. The use of clones in commercial fields have been increasing in the past years. Thus the correct identification of the plants used to clone production would guarantee the genetic gain wished. The aim of this study was identify the origin of clones of Eucalyptus using RAPDs markers. The origins of four clones were determined: clone C7 was originated from matrix M6, clone C6 was originated from matrix M4, clone C14 was originated from matrix M11 and clone C5 was originated from matrix M3. All the clones showed a 100% similarity to the matrix they derived from, but clone C6 that differed from matrix M4 in one locus. This difference may be due to the somaclonal variation detected in micropropagated plants. The RAPD data showed that clones C10 and C11, C15 and C16, which are believed to be originated from different matrix, are originated from matrix C15 and C16.
Financial Support FAPESP

Estimation of genetic variability in selected trees of *Eucalyptus dunnii*

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Species of *Eucalyptus* are widely cultivated throughout the world because of their high wood productivity and adaptability to different industrial purposes. *Eucalyptus dunnii* was introduced into Argentina due to its good adaptation to colder climates (including frost tolerance) and industrial quality of the wood, and has become one of the best adapted species in the pampas. Its original natural distribution is very small, being limited to north-eastern New South Wales and south-eastern Queensland (Australia). A seed orchard is being established in Buenos Aires and agronomic properties as well as genetic similarity between families will be taken into account in its design. To estimate genetic similarity between families, we've used the silver stained AFLP technique on 55 selected genotypes introduced to Argentina in 1988. Similarity matrixes were constructed applying Jaccard index, on the basis of two data sets. The first included all data (monomorphic and polymorphic) totalizing 215 molecular markers, and the second , included only polymorphic markers (140). Average similarity indexes were estimated for each data sets: 0.72"0.051 and 0.42"0.079 respectively. The difference between these was great and the inclusion of monomorphic data considerably increased similarity indexes as expected. Taking into account the fact that polymorphic markers are selected on the basis of their easy reading, they were often underestimated, and monomorphic markers rates, increase considerably. On the basis of these results, it was necessary to define precisely the kind of marker data set that would be used in order to compare different studies and to ensure reproducibility of genetic similarity estimation. Considering that the average polymorphic index content reflected no differences between each of the 4 *Eco*RI-*Mse*I primer combination, a set of extra primers combinations including *Pst*I-*Mse*I were analyzed to select those ones which generate easy scoreable markers. To assess the reliability of this tool for identification of clonal individuals, some clones of 4 different selected genotypes were analyzed and similarities indexes between them raised the identity (value 1) in 3 genotypes and 0.98 (only informative data) in the other one, reflecting the high potential of this technique to be used for vegetable protection purposes. On the other hand, these results will allow to diagram new breeding populations and the introduction of new material in existing populations by selection of germplasm entries. Future QTL analysis for several traits (wood density, frost tolerance etc.) will require the development of useful mapping populations, which are unavailable yet. Controlled pollinations using selected mating pairs with the lowest similarity indexes (0.213-0.250)) will be done..

Paternity determination of superior open pollinated progenies of *Eucalyptus* using microsatellite markers

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In this work we demonstrate the utility of paternity testing of superior half-sib individuals identified in operational forests using highly informative microsatellite markers. A battery of over 20 EMBRA (*Eucalyptus* Microsatellite from Brazil) loci with heterozygosities ranging from 0.80 to 0.95 was used for this work. Individuals were genotyped for a minimum of 12 loci that gave an expected combined power of exclusion well in excess of 99.999%. Two independent sets of 72 open pollinated half-sib individuals were selected for DBH (diameter at breast height) at harvest age in two independent commercial forest stands one at 6 years and the other at 5 years of age. All individuals had DBH > 1.8 s.d. above the mean of the stand. Selection intensity was approximately 3:1000 trees. A set of 72 unselected individuals taken at random were used as controls. All progeny were derived from a seed orchard with a single *E. grandis* maternal progenitor and 6 alleged *E. urophylla* pollinator trees. The reliability of the paternity test in *Eucalyptus* was confirmed using controlled crosses with both parents known. In the first set of selected individuals one was a seed contaminant and one a pollen contaminant. Of the 70 individuals left, 32 (46%) were sired by pollinator tree 1, 11 by tree 2, and 11 by tree 6. Pollinator trees 3, 4 and 5 did not leave any descendants. In the second set of selected individuals four were seed contaminants and 23 were pollen contaminants. Pollinator tree 1 sired 29 individuals, pollinator tree 6 sired 14 individuals and the other two individuals were sired by pollinator trees 2 and 3. In the control sample, 6 individuals were selfs, 14 seed contaminants and 22 pollen contaminants. The interesting result was that pollinator tree 1 sired only 5 individuals while pollinators 2 and 6 sired 8 and 17 individuals respectively. These results taken together indicate that there is great variation in male reproductive success among the 6 pollinator trees in the seed orchard and only three actually left descendants at an appreciable rate. A significant proportion of pollen contamination occurs in the seed orchard. These chance events derived from pollen contamination are unwanted in principle. However they may actually be beneficial when they generate superior individuals with an enlarged genetic base. Pollinator tree 1 was the father of a significantly larger proportion of selected versus non-selected progeny individuals. This results was validated in two independent samples of selected individuals. An immediate recommendation from this analysis is to leave only pollinator tree 1 in the orchard to produce large quantities of seeds. Furthermore selfed individuals should be also selected, grafted and induced to flower to generate potentially superior and uniform hybrid seeds.

Investigation of a putative natural hybrid between *Eucalyptus cloeziana* and *E. acmenoides*

Rhonda Stokoe[1], Merv Shepherd[1], Robert Henry[1], David Lee[1,2] and Garth Nikles[1,3]

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Eucalyptus cloeziana F. Muell (Gympie Messmate) and *E. acmenoides* Schauer. (White Mahogany) have an overlapping distribution in eastern Queensland between Gympie and Cooktown. Putative natural hybrids between *E. cloeziana* and *E. acmenoides* have been observed at two distinct sites, SF944 near Gympie and SF461, south of Cardwell. Taxonomically, such hybrids are of interest as it is the only reported inter-subgeneric hybrids in eucalypts. Currently, *E. cloeziana* is classified into its own subgenus, *Idiogenes*, but has a recognised affinity with the subgenus, *Monocalyptus,* of which *E. acmenoides* is a member. Despite its potential importance, no detailed study to verify this putative hybrid has been undertaken. Molecular, morphological and biochemical techniques are being applied to clarify the taxonomic classification of *E. cloeziana* and its relationship to monocalypts.

Eucalypt genome round-table

Minutes of a meeting held on Friday February 5 1999, commencing at 3:15 in room 400 of the Life Science Building, University of Tasmania

PRESENT :

Chair, Prof Jim Reid (CRC for Sustainable Production Forestry University of Tasmania, Australia)

Dr. Gerd Bossinger (CRC for Hardwood Fibre and Paper Science, The University of Melbourne, Australia)
Dr Margaret Byrne (CALM Science WA, Australia)
Dr Dario Grattapaglia (Laboratorio de Genetica de Plantas. EMBRAPA, Brazil)
Dr Chris Harwood (CSIRO Forestry and Forest Products. ACT, Australia)
Dr Celso Marino (Universidade Estadual Paulista - UNESP São Paulo, Brasil)
Dr David Marshall (Tree Genetics Unit, Scottish Crop Research Institute, Scotland)
Dr Gavin Moran (CSIRO Forestry and Forest Products. ACT, Australia), left early to catch a plane
Dr Brad Potts (CRC for Sustainable Production Forestry University of Tasmania, Australia)
Dr Mervyn Shepherd (CRC for Sustainable Production Forestry, Southern Cross University, Australia)
Dr René Vaillancourt (CRC for Sustainable Production Forestry, University of Tasmania, Australia)
Dr Daniel Verhaegen (CIRAD Forêt, Montpellier, France)
Dr Brenda Wingfield (Forestry and Agricultural Biotechnology Institute, Department of Genetics, University of Pretoria South Africa)

René Vaillancourt acted as Secretary

1. Integration of existing genetic linkage maps with microsatellite markers

The consensus was that most research groups would be using the microsatellites developed by Dr. Grattapaglia's research group for mapping and that linkage group designation in *Eucalyptus* should follow the nomenclature as first published by Dr. Grattapaglia's group.

2. Marker nomenclature

After some discussion it was decided that microsatellite markers should follow the following nomenclature:

EM = *Eucalyptus* microsatellite
followed by a three letter country/lab initials ex. BRA, CRC, CSR, SCO AUS etc.
followed by a unique number (to that country/lab that developed the marker)
Example EMBRA-24 corresponds to a microsatellite developed in *Eucalyptus* in Brazil and is the 24th microsatellite developed by that organisation.

3. Gene nomenclature

The epithet Euc should precede the name used in Arabidopsis, once homologous functionality has been proven. An asterisk should be placed before the Arabidopsis name if gene name homology and/or proof of functionality is weak. These quotation marks can be dropped once better proof is achieved. eg. *EUC*fla.* As in other species, gene name and locus position should be italicised, gene product (RNA and protein) is not italicised.

4. Need for reference pedigree(s) for assignment of markers to linkage groups

Branda Wingfield can make available DNA or tissue cultured plants from a *E. grandis* x *E. grandis* cross to be used as a reference pedigree for assignment of markers to linkage groups

5. *Availability of marker information*

Gavin Moran reported that his RFLP probes are available for exchange upon signing appropriate contracts and that the accessibility of his microsatellite markers is under review by CSIRO.

Dario Grattapaglia has alcv ready made public the sequence of 20 microsatellites, another 40 will be available soon, and possibly 250 by the end of the year.

David Marshall will discuss with Shell the possibility of publishing the sequence of their microsatellite primers.

Daniel Verhaegen has and will publish the sequence of the genes cloned by CIRAD Forêt (France), and primer information is available upon request.

6. *Development of a validated set of SSR loci for fingerprinting applications*

Dario Grattapaglia volunteered to send 12 of his microsatellites primers together with 12 eucalypt DNA samples and information on multiplexing. These samples will be fingerprinted blindly by each participating organisation (so far including; CRCSPF-Hobart, Cirad-France, CALM Science-WA, Scottish Crop Research Institute, Forestry and Agricultural Biotechnology Institute-South Africa, EMBRAPA-Brazil). Each participant will also add 12 unrelated samples from a different species. The outcome of this experiment will be published in Theoretical and Applied Genetics. The significance of this experiment is that this will result in a robust set of microsatellites (transferable across species) for fingerprinting and paternity analysis, that could be used by all researchers working on eucalypt.

7. Make information available in Dendrome database with links to other relevant sites

This database would include microsatellites primer sequences, expected and observed

heterozygosities of microsatellites, linkage maps, and QTL position. The location of this database needs further discussion.

8. Proposals for collaborative projects

A consensus was reached that species of *Eucalyptus* constitute the best **model system** for genome analysis in forest trees, because of its economic importance (compared to poplars), small genome size (compared to pines), and clonability (at least in tropical species). The possibility of launching a project to sequence the entire genome of an eucalypt species, was discussed. It was suggested by some that it may be reasonable to start with a smaller project such as sequencing the entire chloroplast chromosome or 1 Mbb of DNA.

Eucalypt Genome Initiative, is the name that was chosen to define the international collaboration aimed at increasing our knowledge of the genome of eucalypts that was initiated at this meeting.

Close: 4:30pm