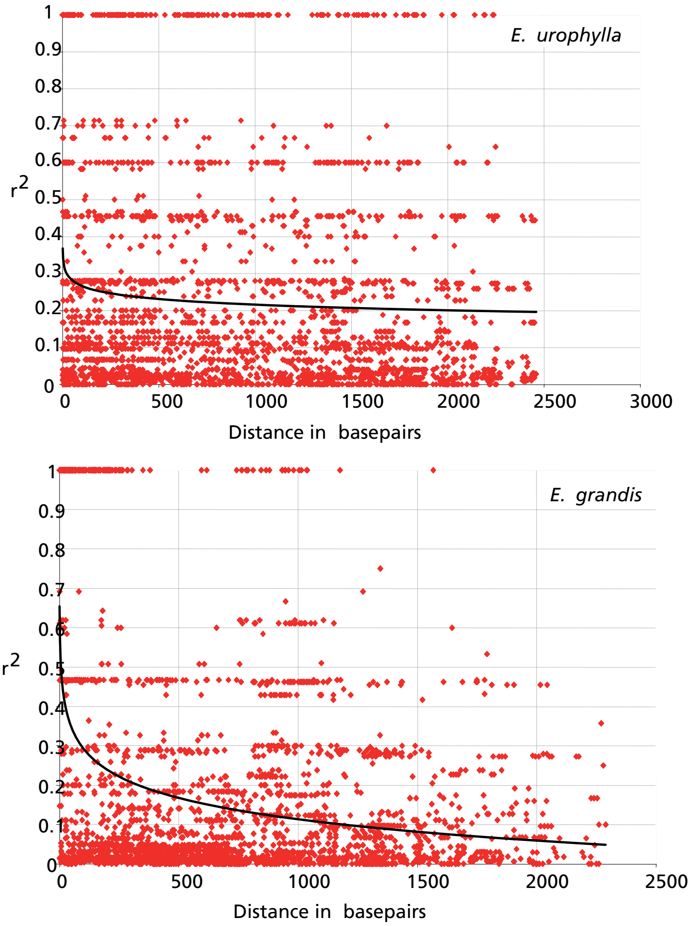
FIGURE 6

**Rapid decay of intragenic linkage disequilibrium estimated as r² in the cinnamyl-alcohol dehydrogenase (cad) gene in two different *Eucalyptus* species**



*Source*: Faria *et al.*, 2006.

LD markers, i.e. polymorphisms that are sufficiently close to the functional mutation (Neale and Savolainen, 2004). The challenge, however, is considerable, as LD in outcrossing forest trees such as pines decays very rapidly, in general within 1 500 to 2 000 bp (Neale and Savolainen, 2004), and similar behaviour has been seen in the few *Eucalyptus* genes analysed to date with significant LD extending for only a few hundred base pairs (Thumma *et al*., 2005; Kirst, Marques and Sederoff, 2005; Faria *et al*., 2006) (Figure 6). Genome-wide association studies for LD marker-trait discovery in tress will require very high SNP marker densities that are currently still impracticable (but see below), so that the only alternative left is a candidate gene approach. Finally, direct markers (i.e. polymorphisms that code for the functional mutations) would be the most valuable and directly applicable in breeding. However, they are the most difficult to detect because causality is very difficult to prove unless very high penetrance Mendelian inheritances are tackled.

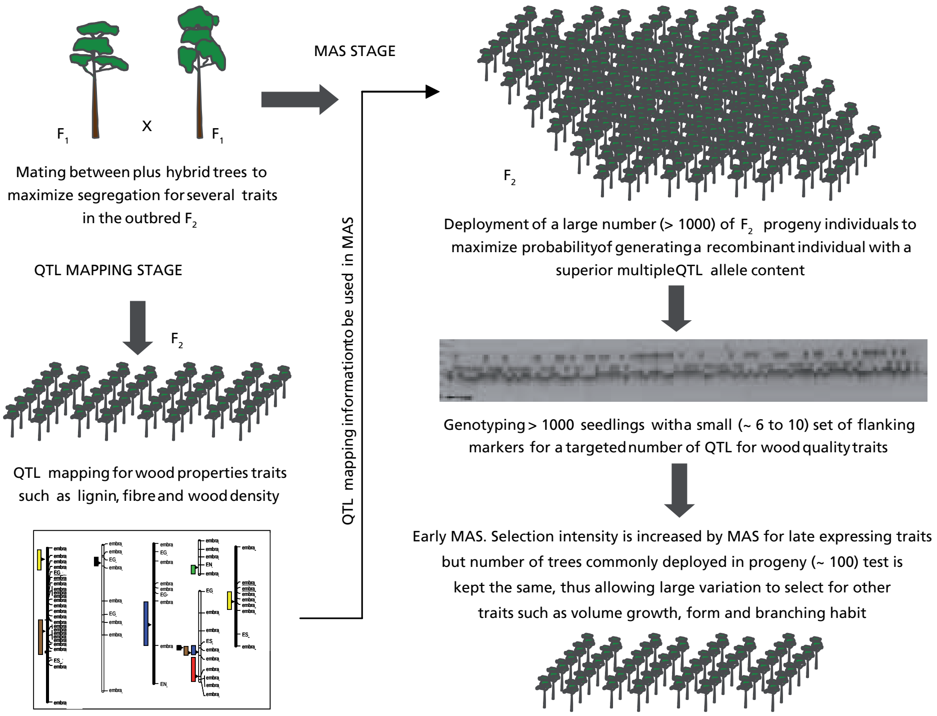
## Prospects for using MAS in *Eucalyptus*

*Eucalyptus* breeding programmes vary broadly according to several aspects including the target species or hybrid, the possibility of deploying clones and the amount of resources available to the breeder. However, from the standpoint of integrating MAS, a reasonable premise is that this will only be a justifiable option when the breeding programme has already reached a relatively high level of sophistication, fully exploiting all the accessible breeding and propagation tools. Advanced breeding programmes that aim at elite clone selection involve a significant amount of time and effort being devoted to clonal testing before effective recommendations can be made concerning new clones for operational plantations. Small subline breeding for hybrid performance combined with clonal propagation of selected individuals is being used increasingly for extracting new elite clones (Potts, 2004). The recombination step of a breeding cycle involves the generation of several segregating progenies from selected parents derived from recurrent selection programmes for general combining ability, or reciprocal recurrent selection programmes for hybrid combining ability. This latter strategy has been adopted in tropical countries where the two reciprocal populations are actually two different species such as *E. grandis* and *E. urophylla*. Controlled crosses that were once an important obstacle for implementing pedigreed selection methods are now used routinely after the relatively recent advances made in controlled pollination methods for *Eucalyptus* (Harbard, Griffin and Espejo, 1999; de Assis, Warburton and Harwood, 2005) (see Figure 2). Progeny trials, together with expanded single family plots where larger numbers of full-sibs per family are deployed, are used to allow very intensive within-family selection based on all the available information, both at the family and individual level using BLUP-based selection indices. This selection is generally carried out at half-rotation age based on growth performance and on a preliminary assessment of wood specific gravity using indirect non-destructive techniques such as pilodyn penetration and/or NIRS and Raman spectroscopy (Schimleck, Michell and Vinden, 1996). Vegetative propagules are then rescued from selected trees either by coppicing, sequential grafting or *in vitro* techniques, multiplied and then used for the establishment of clonal tests.

This breeding scheme generates large amounts of linkage disequilibrium by hybridization and substantial amounts of non-additive genetic variation can be captured by vegetative propagation. These are favourable conditions for MAS in forest trees (Strauss, Lande and Namkoong, 1992). Favourable alleles at QTL segregating within-families could be efficiently tagged with microsatellite markers in linkage equilibrium with the actual functional polymorphisms and used for marker-assisted within-family selection for superior individuals. QTL linked markers could be used to carry out early selection thus reducing the time necessary to carry out the first selection especially for traits related to wood properties, and at the same time reducing the number of trees to be selected, propagated and advanced all the way to clonal trials (Figure 7). Therefore, in the context of molecular breeding, given their relatively short rotations and the possibility of

FIGURE 7

**Proposed scheme for early marker-assisted selection of plus trees to be used as clones**



QTL mapping is carried out and flanking markers in linkage disequilibrium with favourable alleles at major effect QTL alleles are identified. These markers are then used for early within-family selection in expanded progeny sizes at very early age to select for late expression traits such as wood density and lignin content.

deploying clones to capture non-additive genetic variation, it is reasonable to state that eucalypt is the forest tree crop for which MAS has the best prospects of application.

Quantitative theory as well as common sense suggest that MAS in forest trees should help, particularly in situations where trait heritability is low and selection occurs at the level of the individual tree. However, implementing MAS for such traits is a challenging task as extremely precise QTL mapping information is required and this can only be derived from experiments with large progeny sizes (in the order of several hundred individuals), clonal replicates for increased precision, representative and multiple genetic backgrounds and environments. To date, mapped QTL in forest trees still do not fall into this description although improved experiments are under way (Grattapaglia, 2004). Most experiments have mapped QTL for traits that display intermediate to high heritability and probably did not tag the top alleles that exist in the breeding populations as only a very limited sample of crosses were conducted. Furthermore, given the relatively small progeny sizes used for QTL detection (around 100 to 200 individuals), the estimated magnitude of the effects were largely overestimated following the well known "Beavis effect" (Beavis, 1998).

It is frequently stated that MAS for trees would be most useful for volume growth as this is a universal trait of interest and typically of low heritability at the individual tree level. However, in tropical conditions, it is most likely this will not be the target trait of first choice for MAS. Broad sense heritability at the clone mean level, which is the typical selection unit, is frequently above 0.8, allowing an almost perfect ranking and selection of clones even at very early ages (less than two years) under tropical conditions (Rezende, Bertolucci and Ramalho, 1994). Molecular markers for volume growth in these conditions will hardly make a significant contribution to increasing gain per unit time. The cost of scoring molecular markers dictates that the most likely application of MAS in *Eucalyptus* will be for traits that provide significant added value to the final product such as branching habit (for solid wood) and wood chemical traits, or allow clonal deployment such as adventitious rooting or somatic embryogenesis response. Within all possible quality traits, the option would be for those that display medium to high heritabilities but where phenotype assessment is difficult, expensive or requires waiting until the tree reaches maturity. Wood quality traits typically require the tree to start accumulating late wood and involve relatively lengthy procedures for phenotypic evaluation in the laboratory. These kinds of traits could be interesting targets for MAS in *Eucalyptus*, given that the costs of genotyping are sufficiently competitive and precision is high when compared with direct phenotype measurements. It is important to point out, however, that with the recent developments of fast sampling and indirect wood chemistry measurements based on NIRS (Schimleck, Michell and Vinden, 1996), the potential gain will only be realized on the basis of the time savings provided by very early selection. Selected individuals could be recombined more rapidly following flower induction (Griffin *et al.*, 1993) to produce the next generation, potentially increasing the genetic gain per unit time.

MAS for multiple traits will face many of the same difficulties faced by conventional multiple trait selection. Very large progeny sizes would have to be deployed to have a reasonable probability of recovering genotypes with a combination of favourable alleles at many QTL for many traits. When using MAS, priorities will have to be established not only for traits but also for specific QTL. This will require a very good understanding of the relative magnitude of each QTL, potential QTL x background interactions and pleiotropic effects of QTL. Linkage mapping, however, will allow the breeder to understand the basis of negative correlation between traits and possibly to break unwanted linkages by selecting specific recombinant genotypes.

Once the challenging issues related to the discovery of robust marker-trait associations, either within family (LE markers) or at the population level (LD or direct markers), are dealt with, a realistic strategy for the implementation of MAS in *Eucalyptus* might be to tackle only a few major QTL for a quality trait of significant added value. Theoretically, when the total proportion of the additive genetic variance explained by the marker loci exceeds the heritability of the character, selection on the basis of the markers alone is more efficient than selection on the individual phenotype. Such a goal might be achieved for a specific trait with just a few QTL alleles responsible for large effects. On the other hand, if no major gene is detected in an experiment of reasonable size, it might be wiser to dismiss

MAS for that particular trait. Estimates of heritability for a trait might be useful to give an initial clue. Intuitively, the probability of major genes existing for traits of low heritability is lower than for traits of high heritability. However, this should not be taken as a measure to discard possible QTL mapping experiments as, even with low heritabilities, traits might still display major QTL, and MAS would have the greatest impact particularly in such cases.

## CONCLUSIONS AND PERSPECTIVES

The successful application of molecular breeding in *Eucalyptus* will depend heavily on first demonstrating and validating the clear-cut association between a DNA polymorphism and a quantitatively inherited phenotypic trait. In highly heterogeneous eucalypts, while conventional QTL mapping can reveal useful markers to be exploited in within-family selection practices, only a more direct LD mapping approach can uncover population wide applicable marker-trait associations. Such studies based on candidate genes have begun and the first candidate gene association for MFA was detected. However, this association explains only a small proportion (3.4 percent) of the variation to be really exciting news to breeders (Thumma *et al.*, 2005). One of the key issues when embarking on an association mapping experiment is the selection of candidate genes. Maximizing the probability of choosing the proper genes requires levels of knowledge of biochemistry, physiology and development that are generally not yet available even for well defined phenotypes and/or known metabolic pathways.

Following the path taken in human genetics, co-localization of candidate genes and QTL for relevant traits on linkage maps together with integrative expression-QTL mapping (Kirst *et al.*, 2004) could be a powerful way forward, although choosing the correct candidate depends heavily on the precision of the QTL localization. At the moment, there are two possibilities for circumventing the dilemma of choosing candidate genes correctly. The first is microarray-based genotyping with ultra-dense arrays of short (25 nt) oligonucleotides (Borevitz *et al*., 2003; Hazen and Kay, 2003; West *et al.*, 2006) that would allow sufficient throughput for association genetic analysis of thousands of genes at a time. Such an array format could later turn out to be a useful instrument for MAS once validated marker-trait associations have been established. The second would be to have access to a whole genome sequence so that candidate genes in a fine mapping interval delimited by markers flanking a QTL with centimorgan resolution could be mined, reannotated and then analysed in association mapping experiments.

A draft genome of *E. camaldulensis* is currently being sequenced at the Kazusa DNA Research Institute in Japan (T. Hibino, personal communication), and the possibility exists that a fully public 4X draft of the *E. grandis* genome will be sequenced by the Joint Genome Institute of the United States Department of Energy within the next years (J. Tuskan, personal communication) following a proposal recently submitted by an international group of *Eucalyptus* geneticists (www.ieugc.up.ac.za/DOE%20proposal%20-%20final%20-%2026%20July%202006.pdf) who recently formed the International *Eucalyptus* Genome Network (EUCAGEN) (www.ieugc.up.ac.za; Myburg, 2004). Such public collaborative efforts should contribute greatly to the advancement of *Eucalyptus* genetics, genomics and molecular breeding by bringing together existing private databases and genomic resources and thereby expanding the value of such genome sequences. As such genome projects advance and new and more powerful analytical tools become accessible, the true challenge to dissecting the complexity of economically-important traits in *Eucalyptus* and implementing MAS will depend to a large extent on our ability to phenotype trees accurately, analyse the overwhelming amount of genomic data available and translate this into truly useful molecular tools for breeding. MAS should be considered on a case-by-case basis and without overstating the gains to be expected until hard experimental data are accumulated on the actual gains made from its application within industrial forests beyond those which can be attained by comparable investment in conventional phenotypic selection.

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