Opportunities and challenges for the incorporation of genomic analysis in *Eucalyptus* breeding

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Introduction

Molecular marker technologies and forest tree breeding met each other about eight years ago. This encounter was encouraged by the advent of more accessible DNA marker technologies based on PCR (Polymerase Chain Reaction). Since then, DNA markers and tree breeding have been "flirting", many promises of a prosperous union have been made and everyone, especially tree breeders, are still waiting for a happy end or, more appropriately, a happy beginning. This paper, by no means, intends to review the status of applications of molecular markers in forest tree breeding and not even *Eucalyptus* breeding. This would be a challenging endeavor considering that when it comes to applications, several solid programs in the private sector have been established in recent years where confidentiality is rule number one. The aim of this paper is to share some experiences and ideas on what we consider to be the real short term opportunities that exist for the incorporation of genomic analysis in *Eucalyptus* genetics and breeding, and more importantly what are some of the challenges that we still face and can foresee today for implementing some more refined and potentially higher impact applications.

Molecular marker technologies

A molecular marker could be defined as any molecular phenotype derived from a specific genetic polymorphism at a known or anonymous DNA sequence site that could correspond or not to an expressed gene. These polymorphisms are typically detected either by DNA cleavage with restriction enzymes followed by DNA hybridization to specific probes generating RFLP (Restriction Fragment Length Polymorphisms) markers or by DNA amplification with PCR (Polymerase Chain Reaction) driven by specific or arbitrary primers. Useful markers follow mendelian rules of genetic segregation and can display either dominant or co dominant inheritance.

A number of molecular marker technologies have been developed in recent years, particularly after the PCR was described in the late 80's. When invented, each one of these marker technologies received an acronym that described its main features and tried to be appealing to the molecular breeder. As a result, a disconcerting array of marker systems such as RFLP (Restriction fragment Length Polymorphism) RAPD (Random Amplified Polymorphic DNA), AFLP (Amplified Fragment Length Polymorphism), SSR (Simple sequence repeats, also called microsatellites), CAPS (Cleavable Amplified Polymorphic Sequences) and many others are available today for genetic analysis in forest trees. Each marker system is characterized by a number of advantages and limitations that are beyond the scope of this paper but can be found in the literature (e.g. Ferreira & Grattapaglia 1996; Rafalski *et al.* 1996). The main point is that the choice of a marker technology should be driven mostly by the particular application that one has on mind, followed by considerations on the necessary technical skills and facilities, cost and limitations of access to specific reagents and patents and royalties that need to be licensed or paid for.

RAPD markers have contributed significantly to the rapid development of genomic maps and fingerprinting analysis in trees, not only in *Eucalyptus* but also in conifers and Poplars. This was so, as they obviated the need for specific probe development, employed universal "off the shelf" reagents and provided a fast and technically simple entry to segregation, linkage analysis, map construction and genotype fingerprinting. Although concerns were raised on the reliability of the RAPD assay, it has been clearly demonstrated that when appropriate care is taken, high quality maps and fingerprinting analyses can be obtained. The main limitation of RAPD markers is their low information content per locus as they typically display a dominant inheritance and technically only detect one allele at a locus, all the other being pooled in a single class of absence of PCR amplification. They may also display a relatively low transferability from genotype to genotype, although within a population of *E. urophylla* a transferability rate for mapping applications above 60% was estimated (Brondani *et al.* 1996).

AFLP markers share many of the technical advantages and limitations of RAPD. They also employ universal reagents and are dominant as well. Although they are technically more demanding than RAPD markers both in skills and facilities, they have a distinct advantage as they have a much higher multiplex ratio, i.e. the number of genetic loci that can be simultaneously analyzed per single assay, typically a

electrophoresis gel lane. While a RAPD assay in *Eucalyptus* typically surveys between 5 and 10 loci of which between 2 and 6 are polymorphic, an AFLP assay surveys between 100 and 200 genetic loci of which 50 to 100 are polymorphic, depending on the pedigree structure and genetic divergence of the material under study (Gaiotto *et al.* 1997; Marques *et al.* 1997).

SSR (Simple Sequence Repeats) or microsatellite markers are expected to be the most informative class of markers. The distinct value of microsatellite markers arises from their multiallelic nature, codominant inheritance, abundance and wide distribution in the genome and the ability to detect sequence variation by a simple PCR assay, just as RAPD and AFLP. Furthermore markers are in general highly transferable from genome to genome within species and frequently among genetically related species. Apart from the PCR process, they are patent free and sharing of primer sequences among labs should greatly facilitate information flow and comparative studies. The main disadvantage of microsatellite based markers is the relatively high up front costs and necessary molecular biology expertise and infrastructure for the development of specific primer sequences and genetic characterization of allelic variation at amplified loci. Current methods of SSR based marker discovery typically requires genomic library construction, library screening, DNA sequencing of positive clones, primer design, screening of informative loci and characterization of allelic variation. In recent years, the development of SSR based markers has become increasingly accessible mainly due to novel library enrichment strategies and rapid fluorescence-based automatic sequencing technologies (Rafalski *et al.* 1996). Although the cost of development is still high, the broad spectrum of benefits in having such a technology is expected to compensate. Particularly for the genetic analysis of highly heterozygous species of *Eucalyptus*, the availability of PCR based codominant multiallelic markers will become crucial. Most QTL (Quantitative Trait Loci) are likely to be multiallelic so that multiallelic markers will be necessary to map, rank by magnitude of effect and track specific alleles from parents to progeny in marker assisted selection experiments.

Finally, besides using high throughput anonymous marker technologies, the availability of sequence information for large numbers of cDNA will allow the development of PCR based gene markers and the so called transcriptional maps. Knowledge of function of these mapped genes would come from their expected aminoacid sequence and comparison with sequences from reference species such as *Arabidopsis* and others. In theory, using genes as markers instead of anonymous DNA markers such as RAPD, AFLP or SSR would seem to be the best choice as this should facilitate the establishment of putative relationships between candidate genes and specific QTLs mapped in their vicinity, i.e. candidate gene approach. In practice, however, cDNA sequences are much more conserved and therefore detecting polymorphism and allelic variation from individual to individual becomes an arduous task with our current technology making it difficult to use these markers in high throughput analysis to develop linkage and mapping information and use them in marker assisted selection procedures.

Genome Maps and QTL detection in *Eucalyptus*

A number of reports in the literature have demonstrated that DNA markers can be efficiently used to construct high coverage linkage maps of *Eucalyptus* genotypes. These linkage maps have been constructed by co segregation analysis of dominant RAPD markers (Grattapaglia & Sederoff 1994 ; Verhaegen & Plomion 1996) or dominant AFLP markers (Gaiotto *et al.* 1997; Marques *et al.* 1997) in F1 families under a pseudo-testcross configuration, or codominant marker RFLP markers in three-generation outbred F2's (Byrne *et al.* 1995). More recently Brondani *et al.* (1997) have begun a systematic effort to develop a large battery of microsatellite based markers to generate a reference map useful for *E. grandis* and related species of the same subgenus.

For all the *Eucalyptus* maps constructed to date, regardless of the type of molecular marker and pedigree structure employed, the number of linkage groups determined was always equivalent or closely approached the haploid number of chromosomes (n=11) of *Eucalyptus*. The map distance in recombination units was always found to be very close to the estimated total map distance suggesting that genome coverages above 95% were obtained. The total map length was estimated between 1150 cM and 1600 cM. Given an average total map distance of 1300 cM and an average genome size around 650 Mbp/1C the average physical equivalent of 1 cM would correspond to somewhere around 500 kilobase pairs. This relatively small physical distance per recombination unit, when compared to many other plant species and most trees would facilitate physical mapping and positional cloning approaches (Grattapaglia and Bradshaw 1994).

Besides constructing linkage maps several groups have reported the identification of chromossomal regions that have a measurable effect on economically important traits in *Eucalyptus* (Grattapaglia *et al.* 1995, 1996; Byrne *et al.* 1997; Verhaegen 1996; Vaillancourt *et al.* 1995; Shepherd *et al.* 1995) . A variety of molecular marker classes and pedigree types have been used in these experiments. QTLs have been detected in F1, inbred or outbred F2 and half-sib families with or without clonal replicates as well as

exploiting linkage disequilibrium in factorial mating designs. QTL (Quantitative Trait Loci) for juvenile and mature traits have been located both in tropical and temperate *Eucalyptus* species. A common trend, also seen in other plant species, is that a few major loci exist that control relatively large proportions of the total variation in a wide range of quantitatively inherited traits such as volume growth and wood specific gravity.

All QTL mapping studies in *Eucalyptus* have successfully demonstrated experimental approaches for locating QTL on chromosomes and provided some evidence for the numbers and effects of QTLs controlling quantitative traits in commercially important species of this genus. However, due to the relatively small progeny sizes employed, the limited precision for phenotype assessment, the reduced number of genetic backgrounds tested and the inherent limitations of the molecular marker types and pedigree structures used, the true numbers, precise position and particularly the magnitude of effect of QTLs detected for all traits is still insufficiently understood. Currently, several QTL mapping experiments are being repeated with larger sample sizes, improved marker information and pedigree structure not only in eucalypt but also in conifers to better elucidate aspects of the genetic architecture of important quantitative traits in trees.

Showing the possibility of mapping QTLs in a particular pedigree, age and environmental condition does not imply that these QTL alleles will always be important in all genetic backgrounds, ages and locations. Because trees are genetically heterogeneous, it is reasonable to expect that the expression of a particular QTL allele contributed by one parent tree to the progeny may vary depending on the existing genetic background, i.e. the other tree to which it was crossed to. QTL x age and QTL x environment interaction would also be expected considering that juvenile x adult correlations are typically low and significant GXE is the rule in most breeding programs.

Very few studies have been carried out in plants to evaluate aspects of QTL x background and age interaction. This might be so as most QTL mapping studies have been carried out in species with a very narrow genetic base, well known germplasm origin, where inbred lines are themselves or are used to generate the planting stock and where target traits for breeding are simply inherited and involve dominant alleles. Furthermore for crop species age considerations don't exist. Maize, soybean and rice are typical examples. In these conditions QTL x background interaction is not a critical issue. However in maize, Beavis *et al.* (1991) reported that very few plant height QTL were common across F2 populations derived from different inbred lines. In *Eucalyptus*, Campinhos *et al.* (1996) showed that although stable QTL alleles could be detected, a particular QTL allele detected in a particular pedigree might not be as important in all genetic backgrounds. Multiallelism at the QTL, genome dependent expression (epistasis) and sampling effects of insufficient sample sizes of the segregating population are potential explanations for these results. Verhaegen (1996) also reported the detection of some cross specific and some general QTLs when comparing QTLs detected in a single full sib cross with QTLs detected in a diallel mating design. QTL x age interactions were also detected in both studies although in tropical *Eucalyptus* most of the same loci that make a tree grow at age 12 months make a tree grow at age 6 years. Finally, QTL x environment interaction was detected for volume growth when analyzing the same full-sib family clonally replicated in two contrasting environments, although a significant portion of the QTLs had a significant effect in both locations (Grattapaglia, unpub.)

In conclusion, although a significant progress in linkage map construction and QTL investigation in *Eucalyptus* has been made in the last 5 years there is still a lot of information to be developed on the characteristics of specifc QTLs before molecular data can effectively be used into breeding.

Marker assisted selection

The use of molecular markers assisted backcrossing is probably the most concrete application of this technology in crop plant breeding programs around the world. Markers closely linked to the genes to be introgressed from a wild progenitor to a commercial line or from one line to another (backcross line conversion) are used to monitor and select for the presence of those genes in generations of backcrossing. At the same time, selection is also performed based on molecular marker genotypes of the recurrent parent with the objective of recovering the recurrent genome. In *Eucalyptus*, no selection has been reported for any important simply inherited qualitative trait where such an approach would be justified.

Breeders expect marker assisted selection to help particularly in situations where trait heritability is low, typically in selection at the individual tree level. However implementing MAS for such traits is a difficult task as it requires QTL mapping information derived from experiments with large progeny sample sizes, clonal replicates and if possible variable genetic backgrounds. Most QTL mapping studies to date have been carried out with traits that display high heritability and high to intermediate frequency of favorable alleles in the breeding population. These traits can be easily advanced by conventional selection procedures based on phenotype without the need of molecular markers.

Volume growth in tropical conditions most likely will not be a first choice target trait for marker assisted selection. Heritability for growth traits is relatively high in eucalypts so that significant gains can be easily achieved by adopting conventional biometrical approaches given that an adequate amount of genetic variation is available in the breeding population. These gains can be significantly boosted if clonal propagation is used for plantation deployment. Furthermore if clones can be used, the broad sense heritability at the clone mean level is usually around 0.9 allowing an almost perfect ranking of clones even at very early ages (1 to 2 years) in tropical conditions (Rezende *et al.* 1994). Molecular markers for volume growth in these conditions will hardly make a significant contribution to increasing gains.

The expense of scoring molecular markers dictates that the application of MAS with potentially greater impact will most likely be for quality traits, particularly wood quality. Within all possible quality traits, the option would be for those that display higher heritabilities but where phenotype assessment is difficult or expensive. A higher heritability should in principle reflect a smaller number of loci involved and will allow a more precise estimation of location and magnitude of effect of major QTLs. There is probably a better opportunity to justify the cost of MAS for traits that result in a significant added value to the final product such as branching habit (for solid wood), wood chemical traits, adventitious rooting or somatic embryogenesis response. Furthermore, several of such traits are difficult to evaluate either due to the need for the attainment of a certain size or because they require lengthy and costly procedures for phenotypic evaluation in greenhouse or laboratory.

Regardless of trait, it is expected that a significant impact of MAS could be realized in early selection. This could be true both in the context of recurrent selection in small elite populations, as well as for the selection of individuals for vegetative propagation. In recurrent selection, selected individuals could be recombined more rapidly to produce the next generation, potentially increasing the genetic gain per unit time. In the selection of genotypes to be used as clones, the possibility of practicing an intense preliminary selection at a very juvenile stage, would circumvent the common problem of loss of regeneration or adventitious rooting ability with physiological phase change. Pre-screening of candidate genotypes would be carried out based on favorable QTL alleles composition, before they enter the clonal testing phase potentially reducing the costs of clonal trials. For species that have rooting problems, selected individuals could then be micropropagated or kept as juvenile hedges immediately. In a first stage of this practice, results of clonal trials in the field would be needed to corroborate the preliminary selection. In later stages of the program, once validation and prediction experiments had been accomplished, a strict selection on QTLs could be implemented and selected individuals could be immediately managed to be deployed as clones.

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

A realistic strategy for the implementation of MAS might be to tackle only a few major QTLs. Theoretically, when the total proportion of the additive genetic variance explained by the marker loci exceeds the heritability of the character, selection on the markers alone is more efficient than selection on the individual phenotype. It might be possible that for a certain trait, with just a few QTLs with large effects, such a goal is achieved. 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 existing major genes 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. Even with low heritabilities, traits might still display major QTLs, and particularly in those cases MAS would have the greatest impact.

Management of genetic variation in breeding and production populations

While the use of markers for selection of superior genotypes is still a challenge that depends on further and more refined experimental work, molecular markers can be immediately used to solve several questions related to the management and identification of genetic variation in breeding and production populations. This is particularly true in eucalypt where several breeding programs are still at the stages of establishment of long term breeding populations. Furthermore, advanced breeding in *Eucalyptus* tends to minimize the need for controlled pollination due to the great expense and time usually needed to complete large mating designs. In such incomplete pedigree designs, markers can be efficiently used to monitor coancestry build up

in generations of selection. High throughput molecular marker technologies allow a very efficient genome sampling and precise estimation of genetic divergence, variability and discrimination among genotypes. Some short term applications of molecular markers for management of genetic variation in *Eucalyptus* can be outlined as follows:

- Characterize large base populations by quantifying the levels and organization of genetic variation within and between provenances and progenies. These data can be immediately used to structure new breeding populations, infuse new material in existing populations and decide on maintenance, enrichment or elimination of germplasm entries;
- Resolve questions of clonal identity in seed orchards management and variety protection;
- Generate information on genetic origin, divergence and relationship among elite hybrid clones of unknown origin for defining deployment strategies and mating schemes;
- Estimate outcrossing rates in clonal orchards and/or breeding populations advanced by incomplete pedigree. Information on the predominant system of mating is essential as it ultimately regulates the distribution of genetic variation between and within o.p. families and determines the rate of increase in coancestry;
- Optimize the resource allocation for controlled crosses in reciprocal recurrent selection programs by identifying more divergent crosses. Correlation between divergence measured at the DNA level and mean progeny performance for quantitative traits still needs to be clearly established in *Eucalyptus* and should not be assumed a priori.
- Incorporate genetic diversity estimates for phenotypically top ranked families at selection time, aiming at the maximization of genetic gain coupled to the maintenance of maximum levels of genetic diversity for future generations of breeding.

Conclusions and perspectives

Technology and cost-wise we anticipate the development of increasingly faster and cheaper marker technologies, including DNA chips that would allow the simultaneous analysis of thousands of genetic loci by digital scanning. This technology advancement will be followed by the establishment of an increased number of private labs providing genotyping services at very competitive costs and high quality. In a few years, the challenge will not be to generate marker information but rather to be creative at knowing exactly what information to buy and how to use it. Improved statistical analysis tools for molecular marker data have been developed in recent years and are becoming further refined allowing the analysis and accommodation of several types of genetic and QTL mapping approaches as well as simulation of marker assisted selection schemes (Liu 1997).

Aware of the kind of information that molecular markers can provide, and the costs associated, the question that remains is whether there are real opportunities for incorporating molecular technologies in one's *Eucalyptus* breeding program. Clearly, markers might be used as a tool to manage genetic variation. This is a ready-to-go technology and applicable in several operational procedures both in beginning and advanced programs. Although useful, this might not be a priority for programs still in their inception, run on very tight budgets and where significant gains can be achieved by implementing conventional selection procedures coupled to cloning.

In advanced breeding programs, markers could become useful for tracking the inheritance and introgression of favorable alleles. At this stage, when favorable alleles of intermediate and high frequency have been exploited, variation becomes limiting and progress will depend on fine tuning genotypic combinations by altering frequencies of rare QTL alleles of large magnitude of effect. MAS would be most likely applied in the improvement of the multiple elite populations developed specifically for extreme quality trait values. This effort should target the most valuable individuals in a breeding population, and probably begin by working with only one trait in a specific breeding group or in a special MAS elite population. MAS would hardly be justifiable for improvement of main breeding populations both due to the large number of individuals involved and the very general objectives of selection that are adopted.

The effective incorporation of MAS in *Eucalyptus* breeding clearly requires significant further experimental work. To this end it is essential that large sample size, clonally replicated experiments in several locations and genetic backgrounds be established. It is important to point out that the fact that a particular QTL allele is not detected in all genetic backgrounds does not mean that the locus is not there. It suggests that other better alleles at that locus occur in the population. The challenge for molecular breeders is to discover and rank those alleles. To carry out such ranking, transferable multiallelic microsatellite markers will be a must. Only by tracking alleles of large effects will MAS effectively contribute to a quickest advance of breeding programs. Although a number of QTL mapping experiments are underway around the world, they are certainly not enough to supply definite and quick answers to all the main challenges that exist for the

implementation of MAS. Because MAS has to be considered on a case-by-case basis both in terms of target traits and material available, any forestry company that wishes to adopt molecular breeding, should plan to devote a substantial effort in R&D either in house or through pre-competitive research cooperatives to evaluate the potential of the technology in applied eucalypt breeding.

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