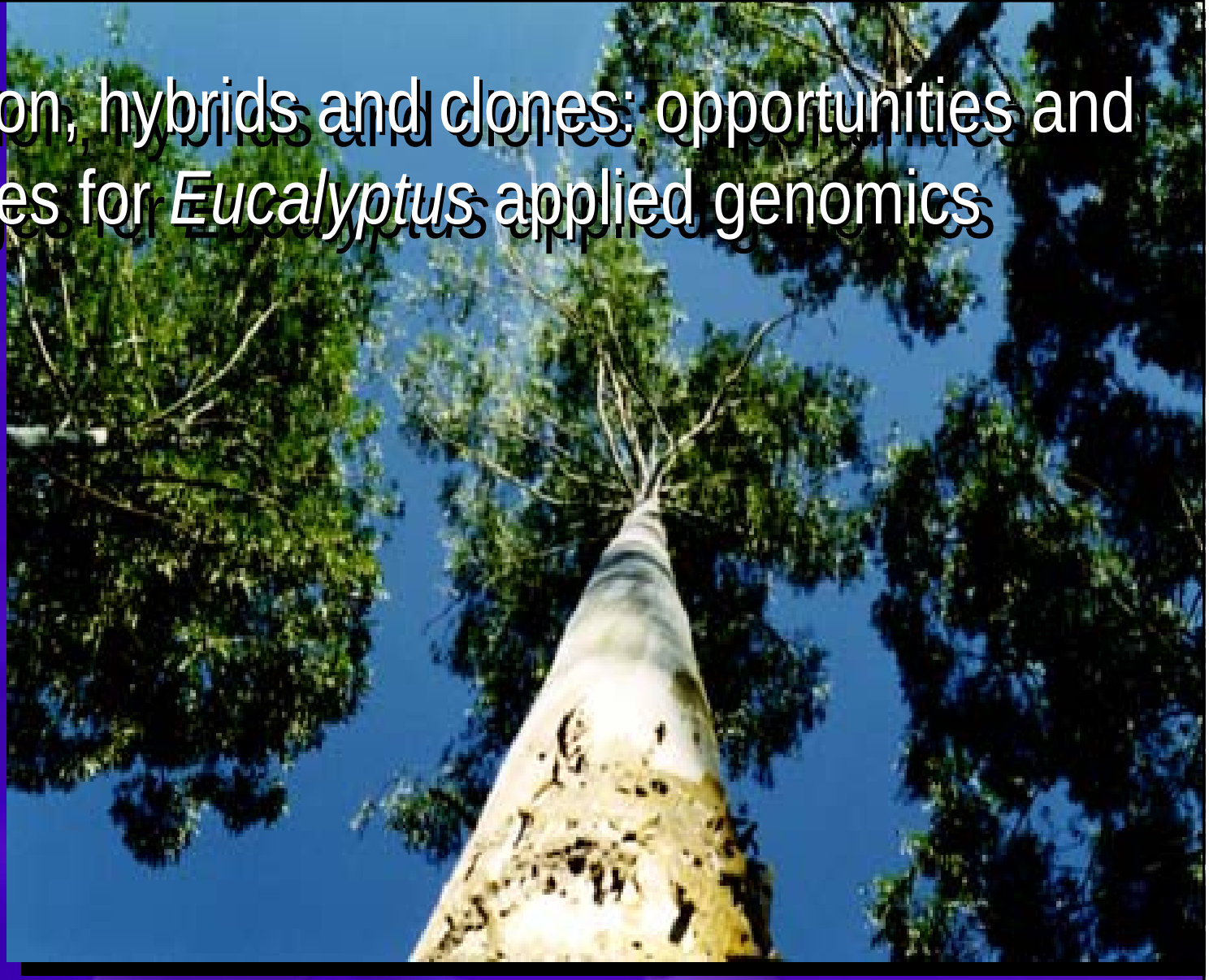
Natural variation, hybrids and clones: opportunities and challenges for *Eucalyptus* applied genomics



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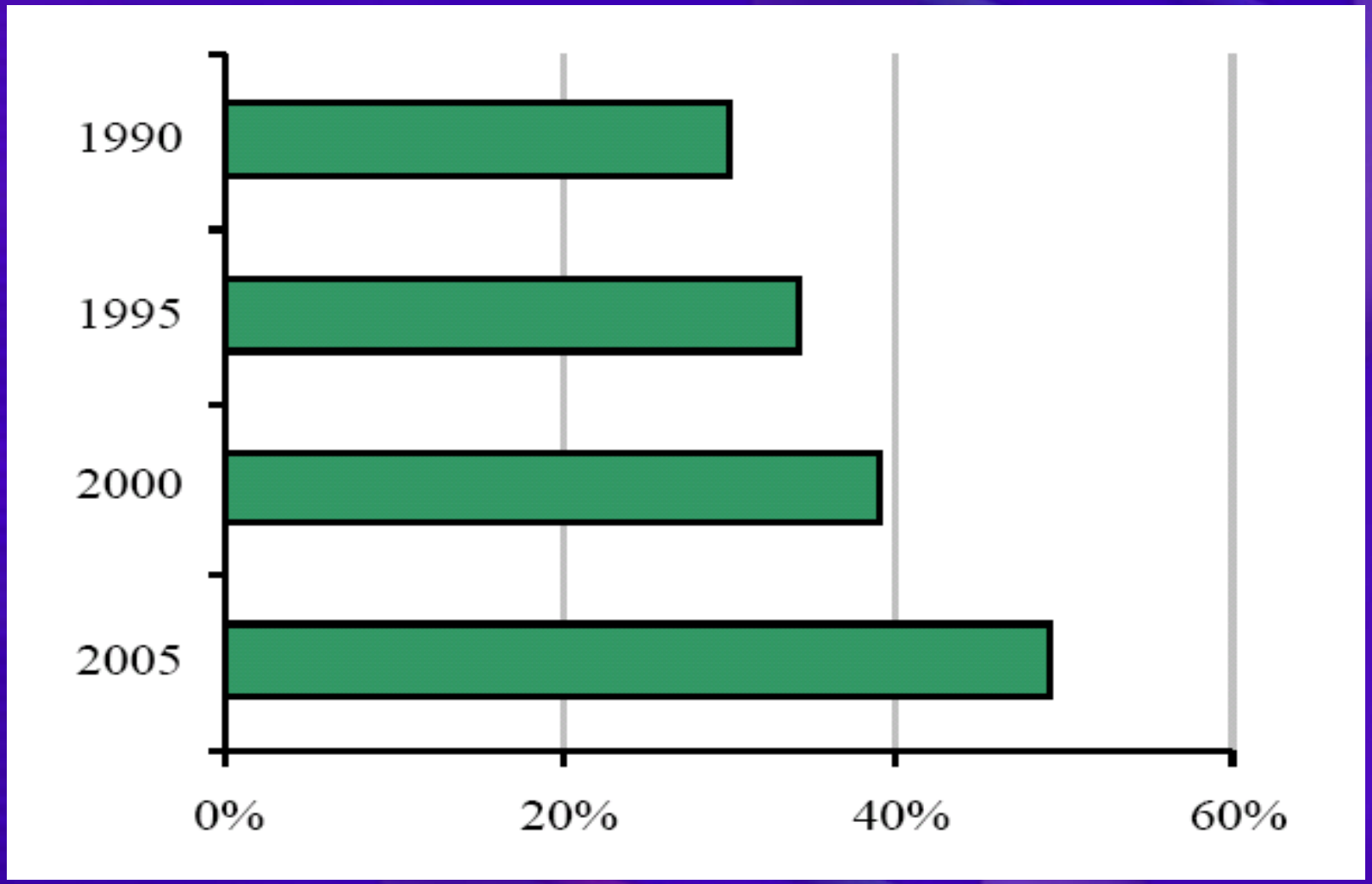
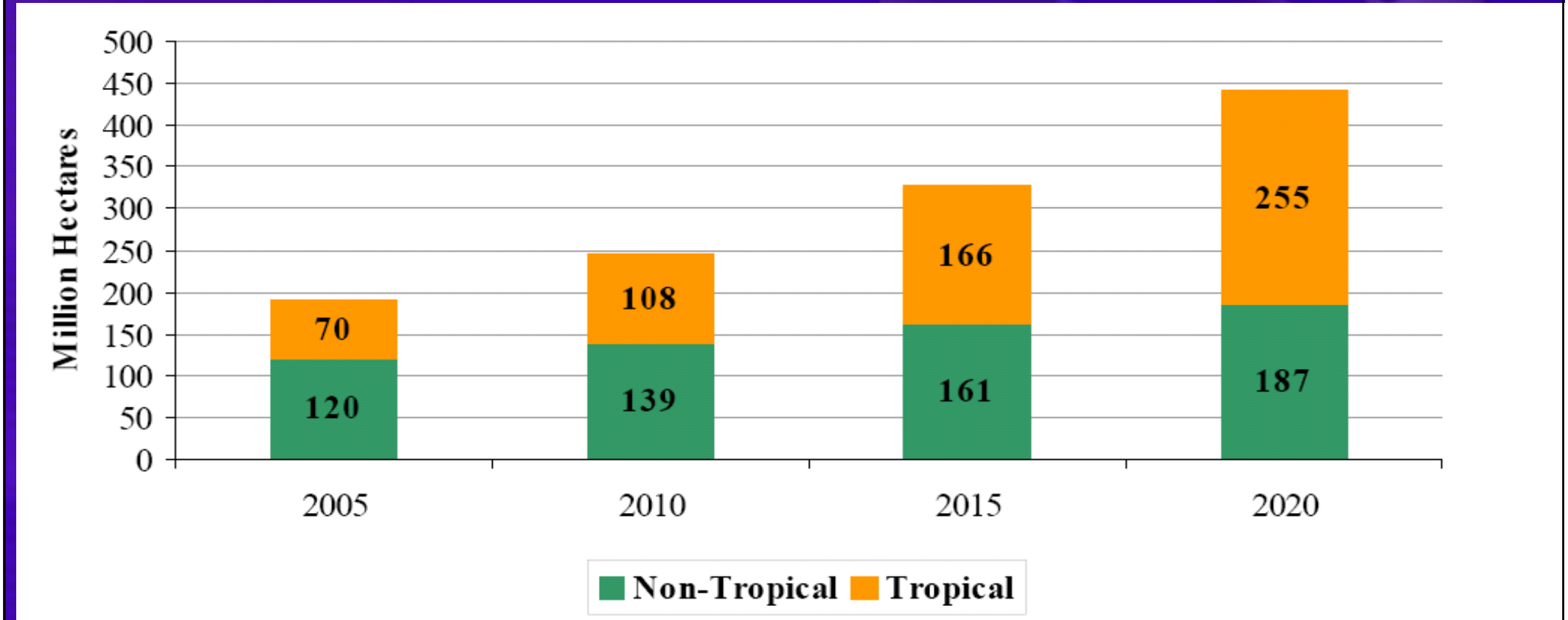
✓ Genomic Sciences Program - Universidade Católica de Brasília

Share of *Eucalyptus* in the international trade of short fiber wood pulp



Source: BRACELPA, 2007

Projected world forest plantation area



- ✓ Eucalyptus is currently the most productive and main planted species in the tropics
- ✓ Key species for pulp, potential for solid wood and growing productivities and quality
- ✓ By 2020 Eucalyptus pulp will have over 60% of the international trade of short fibre pulp
- ✓ Also eucalyptus logs from plantations will have a larger share in the sawnwood and plywood industry, and will take market shares of tropical timber from natural forests

Source: FAO , GLOBAL WOOD AND WOOD PRODUCTS FLOW 2007

Eucalyptus industrial forests in Brazil

- Fast growing eucalypt forests
  - ~4 million hectares, 40% of the intensively world planted Eucalyptus area
  - Main supplier of woody biomass with specific wood properties for several industrial activities (pulp and paper, steel, solid wood products)

- Main competitiveness factors:
  - Genetic material: adaptability and variability
  - Advanced breeding programs for wood quality
  - Clonal forests – high selection intensity
  - Silvicultural practices
  - Growing critical mass in R&D both in industries and universities

Forest based industrial products in Brazil

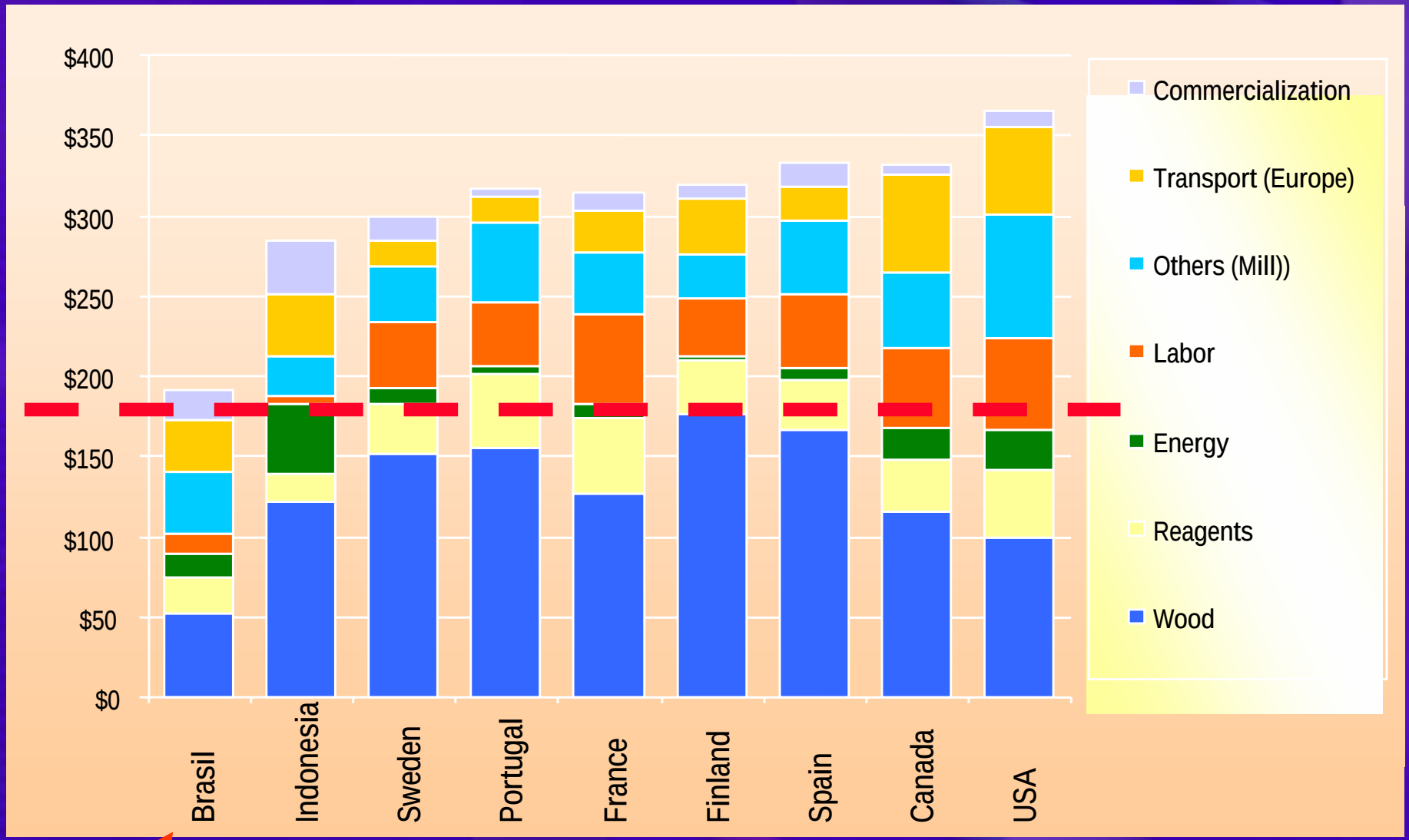
Product	UN 1000	Production	Domestic Consumption	Source	
				Planted(1)	Native(2)
Pulp	t	8.020	5.020	100%	-
Paper	t	7.800	6.879	100%	-
Charcoal	mdc	26.200	26.200	68%	32%
Sawnwood	m^3	22.300	20.000	35%	65%
Plywood	m^3	2.600	900	60%	40%
MDF	m^3	845	716	100%	-
Particle Board	m^3	1.800	1.800	100%	-
OSB	m^3	90	80	100%	-
Fiberboard	m^3	507	295	100%	-
EGP	m^3	285	220	100%	-
Mouldings	m^3	490	50	40%	60%
Doors	um	6.300	4.700	70%	30%
Floors	M^2	22,50	15,20	50%	50%
Blocks / Blanks	m^3	430	360	100%	-

(1) Industrial roundwood: 110 millions m^3

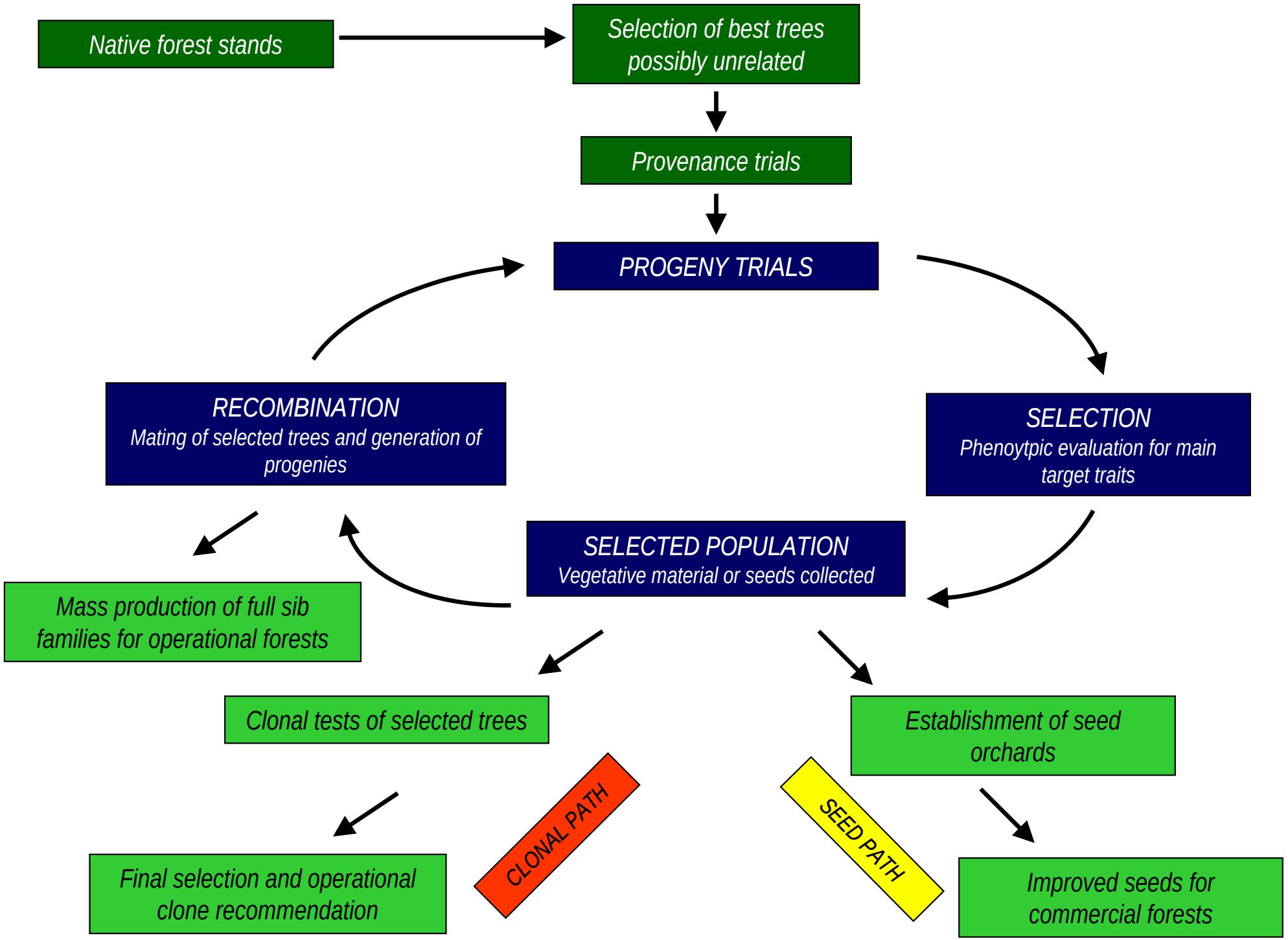
(2) Industrial roundwood: 66 millions m^3

Source: Sociedade Brasileira de Silvicultura, 2003

Production and commercialization costs of short fiber pulp in several countries in 2002 (US$/ton)



Source: Hawkins Wright-11/02


Native forest stands
Selection of best trees possibly unrelated
Provenance trials
PROGENY TRIALS
RECOMBINATION
Mating of selected trees and generation of progenies
SELECTION
Phenoytpic evaluation for main target traits
SELECTED POPULATION
Vegetative material or seeds collected
Mass production of full sib families for operational forests
Clonal tests of selected trees
Establishment of seed orchards
CLONAL PATH
SEED PATH
Final selection and operational clone recommendation
Improved seeds for commercial forests

The tree is the real pulp mill

- ✓ Increased forest productivity: m^3 wood/hectare/year
- ✓ Reduction in wood specific consumption for pulp production: m^3 of wood/ton of cellulose pulp
- ✓ Combination of large wood volume per hectare with low specific consumption of wood per ton of pulp: reduced industrial costs
- ✓ *Breeding is key to competitiveness*
- ✓ *Genomics will be one more tool available to the breeder*

Economic value of volume growth x pulp yield in *Eucalyptus*

- ✓ Wood density and pulp yield are highly correlated
- ✓ Wood density and pulp yield are 3X more important than volume growth in the final cost of cellulose pulp
- ✓ Gains of 10% in wood specific consumption (ex. reducing from 3.6 to 3.24 m^3/ton pulp) corresponds to a gain of 30% in volume growth (ex. going from 35 to 50 m^3/ha/yr)
- ✓ Gains are made along the whole production chain (harvesting, transportation, yearly mill efficiency etc.) as these make up 2/3 of the final cost of pulp production

(SOURCE: Borralho et al. 1993)

Interspecific variation for wood quality traits in *Eucalyptus*

ESPÉCIE	AMI m^3/ha/ ano	PULP YIELD (%)	DENS (g/cm^3)	S5 (%)	SPEC. CONS. (m^3/ton)	LIGNIN (%)
E. grandis	**42**	50	0,42	10	4,7	25,5
E. tereticornis	25	46	0,56	9	3,8	30
E.saligna	36	49	0,46	9	4,4	26
E. urophylla	30	49	0,48	11	4,2	28
E. globulus	25	**54**	**0,55**	**15**	**3,3**	**22**

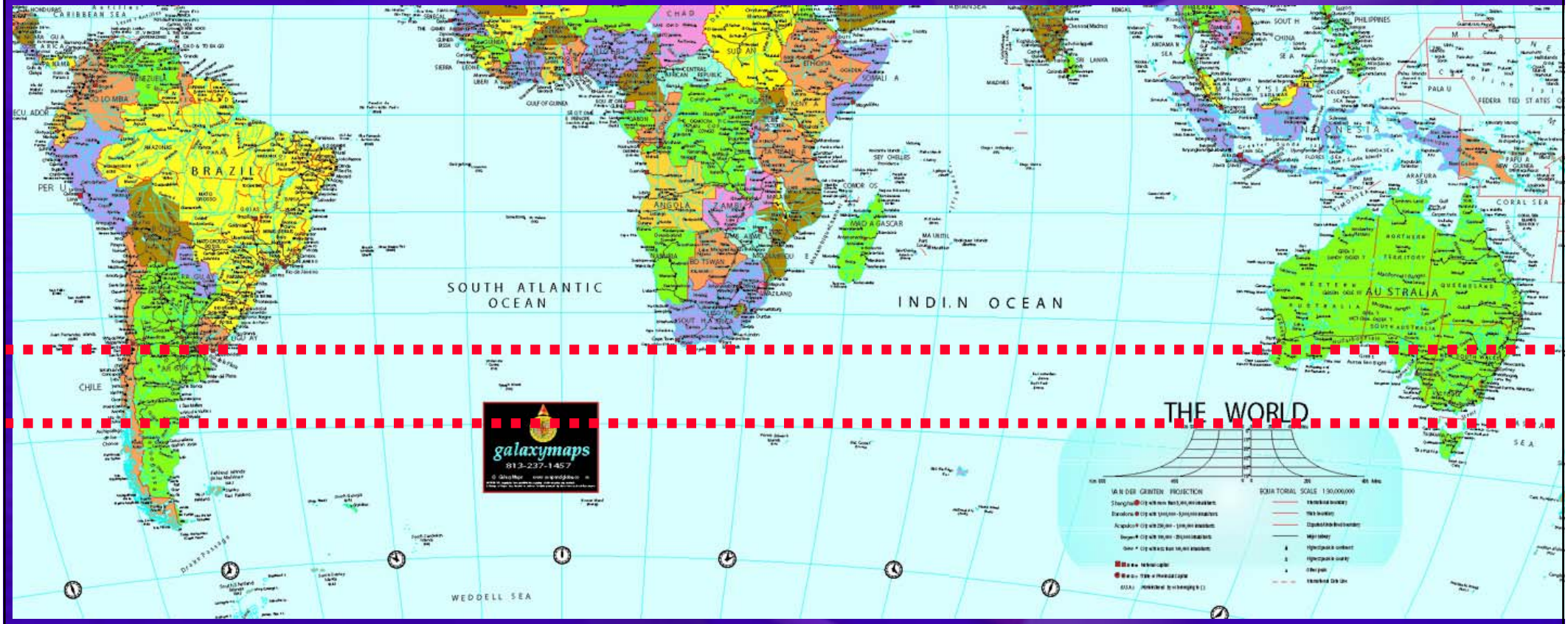
Eucalyptus globulus:

Paradigm of wood quality for pulp

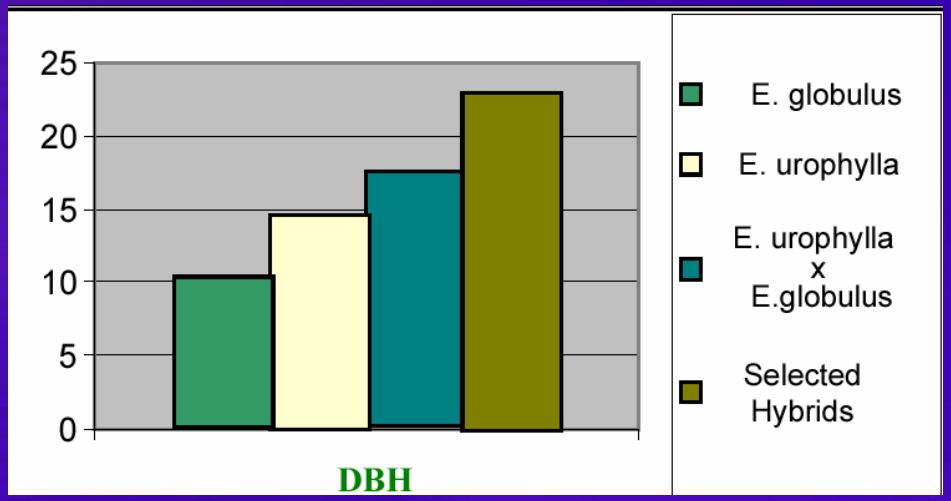
- ✓ Best combination of wood properties for pulp and paper
- ✓ Requires ~25% less wood per ton of cellulose
  - ✓ *E. grandis*: 3.89 m^3/ton of cellulose
  - ✓ *E. globulus*: 2.98 m^3/ton of cellulose
- ✓ Longer and thicker fiber
- ✓ Larger holocellulose content
- ✓ Better fiber flexibility
- ✓ Basic density around 550 kg/m^3
- ✓ Requires less energy in the industrial process
- ✓ Better fiber preservation during the pulping process

Wood properties and adaptation to the tropics

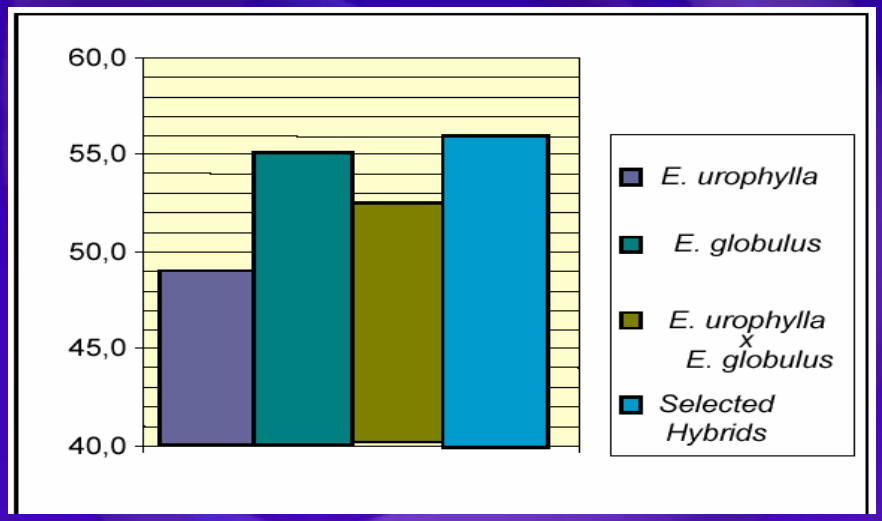
- ✓ *E. globulus* is adapted to latitudes below 33ºS and clearly unadapted to less than 28ºS
- ✓ Evidences exist that wood anatomy co-evolves with latitude and tropicality in response to high demands of transpiration and possibly disease resistance (e.g. Jansen et al. 2004; Noshiro and Baas 2000; Swenson and Enquist 2007).
- ✓ This variation seems to be related mostly to vessel structure and less to fibers, the most relevant elements to wood quality
- ✓ Good perspectives of introgressing the exceptional wood traits of *E. globulus* into tropical Eucs



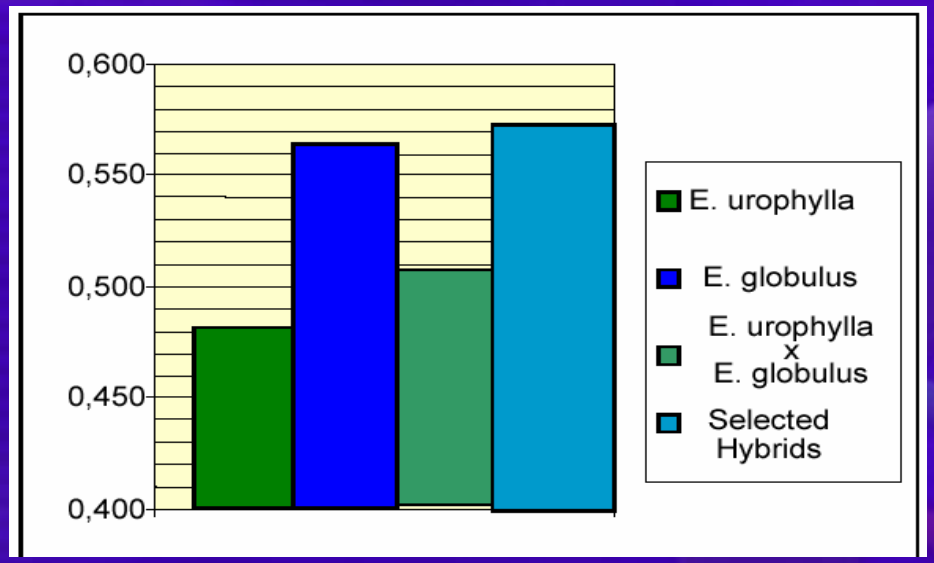
Introgressing *E. globulus* genes into tropical *Eucalyptus*: gene action for wood traits is mostly additive



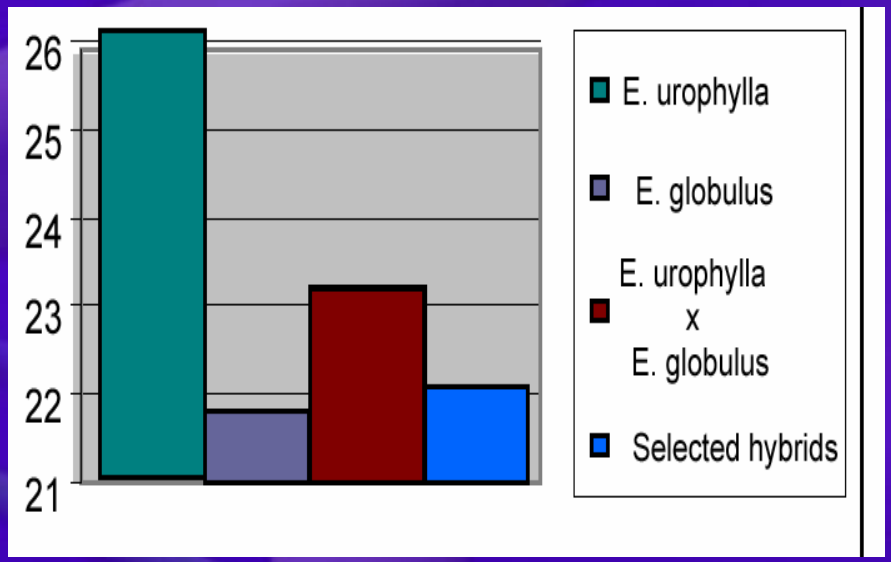
Volume growth



Cellulose yield



Wood specific gravity



Total lignin content

Data from Teotônio F. De Assis Klabin , 2003

PERFORMANCE OF SOME OF THE FIRST GENERATION *E. globulus* TROPICAL HYBRID CLONES

E. globulus hybrids						
CLONE	DENS. Kg/m³	P.Y. %	Sp. Cons. m³/tsa	S_S %	Visc. cm/g	LIG. %
8067	606	55,7	2,96	13,5	1040	22,3
8064	561	56,6	3,15	12,8	1070	22,2
8084	594	54,7	3,08	14,3	1110	22,7
8063	574	57,5	3,02	13,2	1020	21,0
8069	584	56,5	3,06	15,1	1120	21,4
8047	600	55,8	2,98	14,3	1020	21,4
8095	602	55,3	3,10	12,8	1000	23,6
8056	565	55,0	3,21	14,6	1020	22,2
8057	628	55,2	2,88	13,7	1010	22,3
8046	582	55,4	3,10	14,1	1030	21,8
Average	**590**	**55,6**	**3,05**	**13,8**	**1040**	**22,7**
E. globulus	**550**	**54**	**3,3**	**15**	**1100**	22

Data from Teotônio F. De Assis – Klabin 2003

E. urophylla x E. globulus hybrid in Minas Gerais – Cenibra 2006

GRANDIS X GLOBULUS

WHY ARE THEY SO DIFFERENT ??

- ✓ Contrasting species are genetically and genomically very close, can be easily interbred and generate fertile intermediate hybrids
- ✓ What are the genetic and molecular bases of the observed phenotypic differences
  - ✓ Differences in coding regions: enzymes?
  - ✓ Differences in regulatory elements: cis, trans, microRNA?
  - ✓ By understanding these differences it should be possible to exploit the natural variation in a more directed way through both molecular and conventional breeding

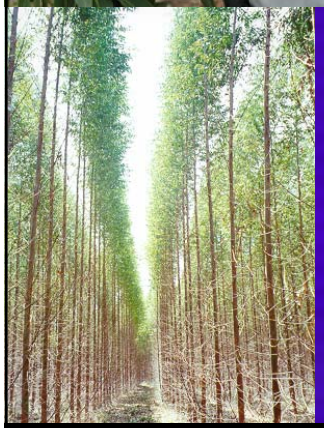
Large scale phenotyping in segregating populations

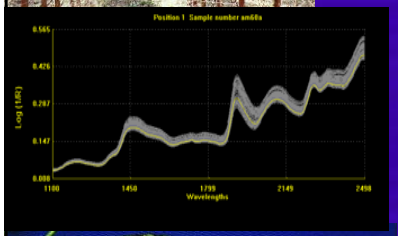
Genetic map of transferable markers and QTLs

High resolution genetic map (SNPs)

Minimum tiling path physical map (BAC *Fingerprinting)*

Identification and validation of target genes





Cross validated QTL LOD >>3,0

LOD = 3,0

~ 10 cM

< 1,0 cM

~ 500 a 1000 kpb

Genetic mapping of candidate genes

Association mapping (*whole genome scan*, candidate genes)

Full genome sequence assembly

Functional tests by transformation (complementation, superexpression, interference)

Forward genomics approach

from phenotypes to genes

GENOLYPTUS *forward* genomics strategy:

from phenotypes to genotypes

Growth, flowering

Disease resistance

WOOD PROPERTIES

FIELD EXPERIMENTS

- ✓ 22 connected full sib families amongst divergent trees
- ✓ Large progeny sizes (> 1200 individuals)
- ✓ Partial clonal replication
- ✓ Several genetic backgrounds and locations

PHYSICAL MAPPING

- ✓ BAC library
- ✓ Anchoring with genetic map
- ✓ BAC end sequencing
- ✓ BAC shotgun sequencing
- ✓ Full gene identification
- ✓ New microsats development

PHENOTYPES

LINKAGE AND ASSOCIATION MAPPING

QTL & candidate genes

GENES & LINKED MARKERS

GENE DISCOVERY

- ✓ Several cDNA libraries
- ✓ EST sequencing
- ✓ Bioinformatics: data mininig
- ✓ Microarrays
- ✓ Candidate gene mapping -SFP

QTL MAPPING

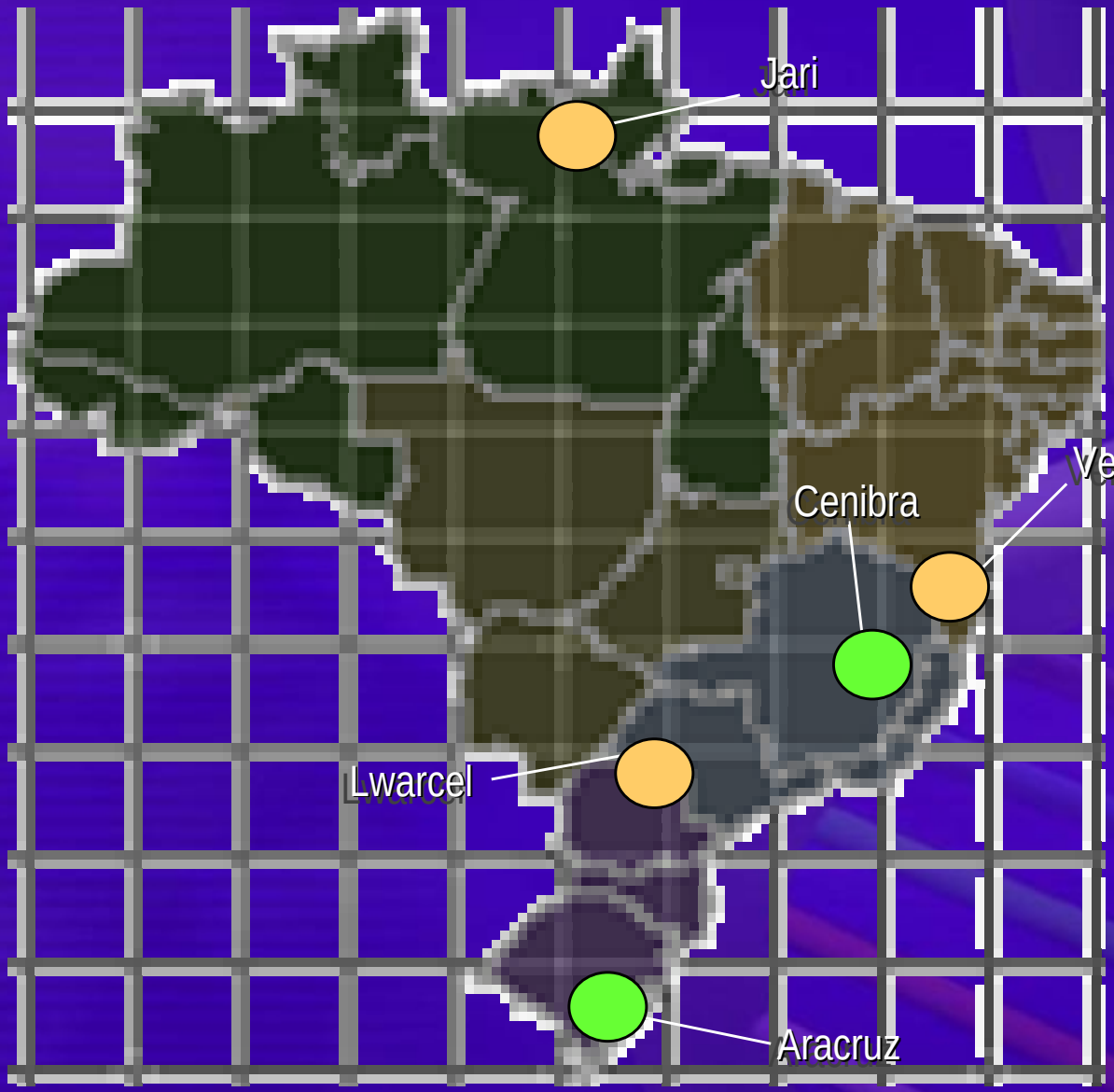
- ✓ Genetic map construction in multiple families
- ✓ Genome scan with 200+ microsats in multiplexes
- ✓ QTL detection & validation

Mating design used to generate segregating families to understand wood properties and adaptation and map QTLs

	E. grandis Atherton Aracruz	*E. urophylla* Timor Cenibra	*E. globulus* K-Riocell	*E. dunni* K-Riocell	*E.camaldulensis* V-Mannsmann	*E. uro.* x *E. glob.* K-Riocell
E. grandis *Coffs Harb.* VCP	G1 x G2 (est. VCP x pól. AR)	G1 x U2 (est. VCP x pól. CE)	G1 x GL2 (est. VCP x pól. K-R)	G1 x D2 (est. VCP x pól. K-R)		G1 x (UxGL) (est. VCP x pól. K R)
E. urophylla (Flores) IP	U1 x G2 (est. IP x pól. AR)	U1 x U2 (est. IP x pól. CE)	U1 x GL2 (est. IP x pól. K-R)	U1 x D2 (est. IP x pól. K-R)	U1 x C2 (est. IP x pól. V-M)	U1 x (UxGL) (est. IP x pól. K-R)
E. globulus K-Riocell	G2 x GL1 (est. AR x pol. K-R)	U2 x GL1 (est. CE x pól. K-R)			C2 x GL1 (est. V-M x pól. K-R)	
E. dunni Rigesa	D1 x G2 (est. RG x pól. AR)	D1 x U2 (est. RG x pól. CE)	D1 x GL2 (est. RG x pól. K-R)	D1 x D2 (est. RG x pól. K-R)		D1 x (UxGL) (est. RG x pól. K-R)
E. camaldulensis V-Mannesmann	G2 x C1 (est. AR x pól. V-M)	U2 x C1 (est. CE x pól. V-M)	C1 x GL2 (est. V-M x pól. K-R)	C1 x D2 (est. V-M x pól. K-R)	C1 x C2 (est. V-M x pól. V-M)	C1 x (UxGL) (est. V-M x pól. K-R)
E. gran. x *E. dunni* K-Riocell	(GxD) x G2 (est. K-R x pól. AR)	(GxD) x U2 (est. K-R x pól. CE)	(GxD) x GL2 (est. K-R x pól. K-R)	(GxD) x D2 (est. K-R x pól. K-R)	(GxD) x C2 (est. K-R x pól. V-M)	(GxD) x (UxGL) (est. K-R x pól. K-R)

Crosses made with elite parents from different companies

Field experiment network of the GENOLYPTUS project: *planted in 07-10/2003*



Main experiments

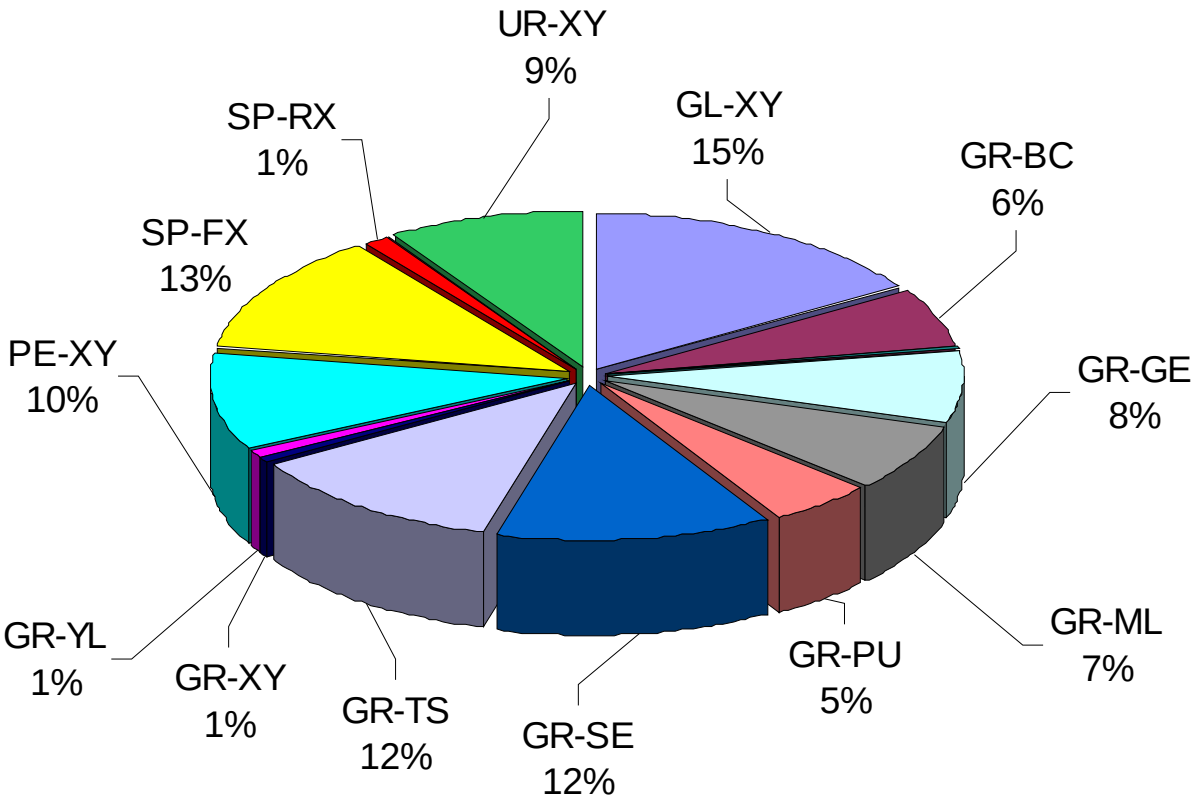
Complementary experiments

✓Planted 22 segregating families for QTL mapping and validation

✓Over 1000 trees per family for 13 families

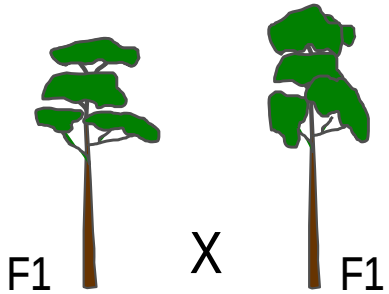
✓Several hybrid combinations involving *E. globulus*

Genolyptus libraries



GENOLYPTUS EST database

- ✓ *4 species*
- ✓ *11 libraries*
- ✓ *124,851 reads*
- ✓ *21,442 consensi*

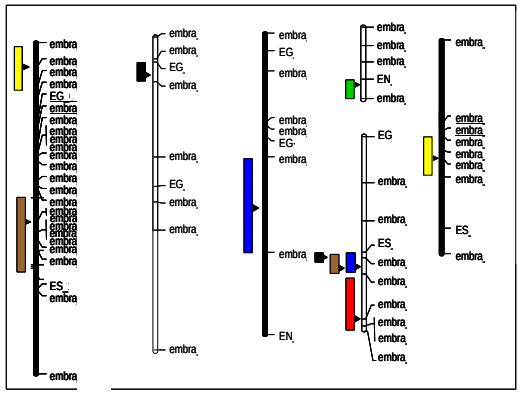


Mating between plus hybrid trees to maximize segregation for several traits in the outbred F2

QTL MAPPING STAGE

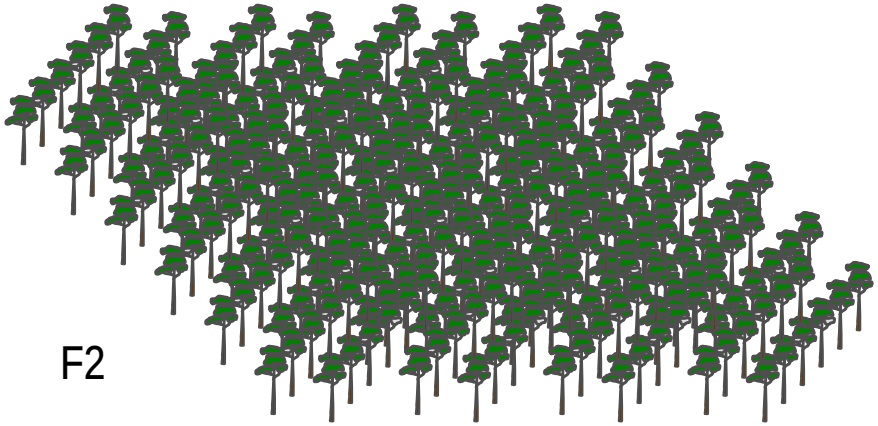
F2

QTL mapping for wood properties traits such as lignin, fiber and wood density



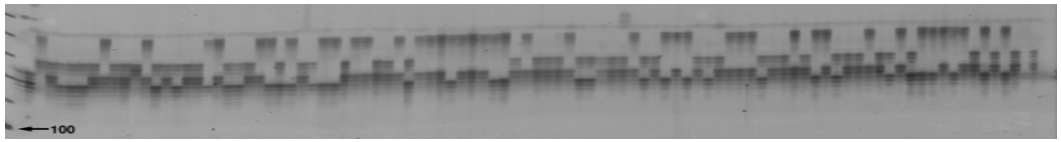
QTL mapping information to be used in MAS

MAS STAGE



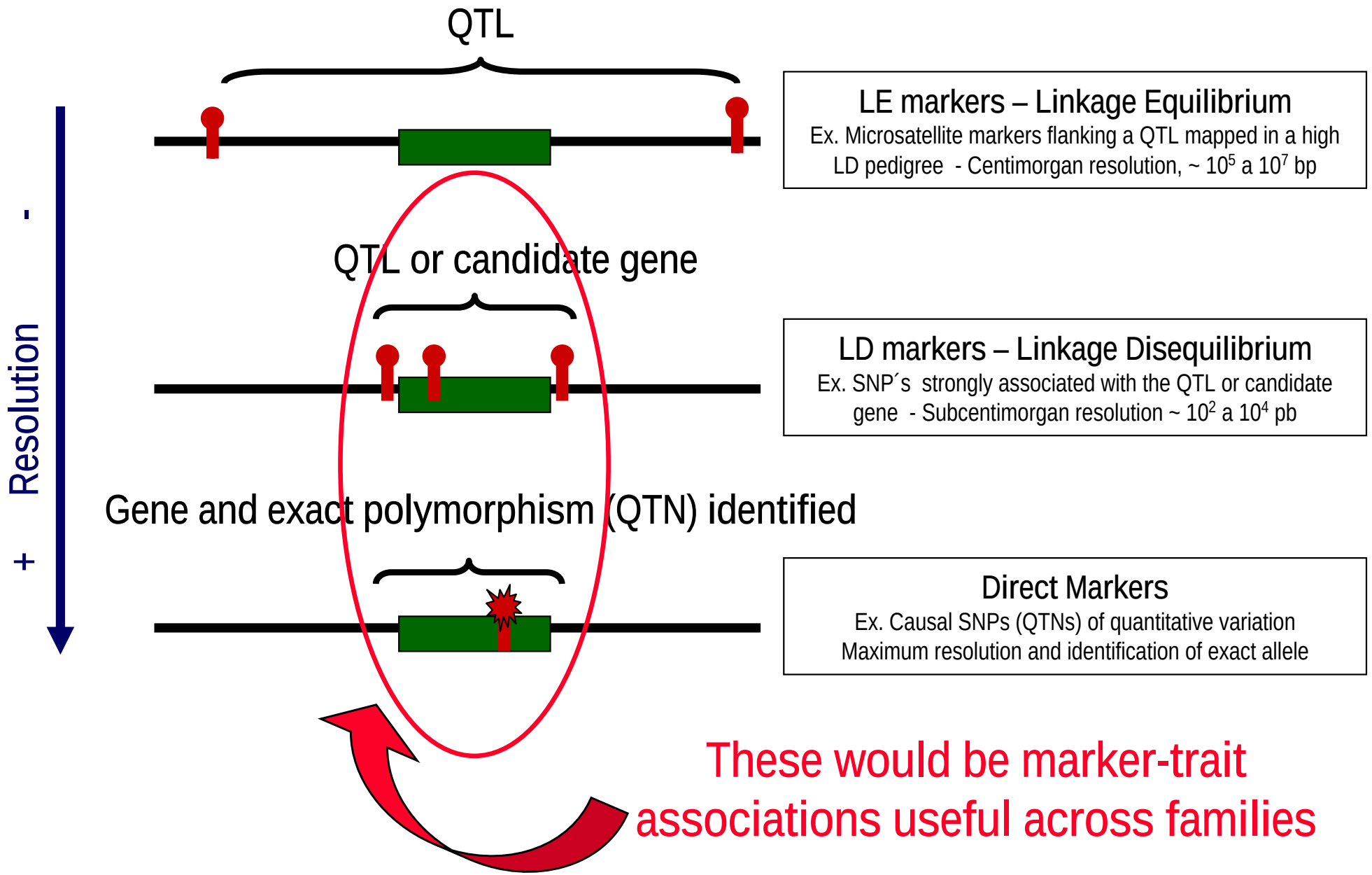
F2

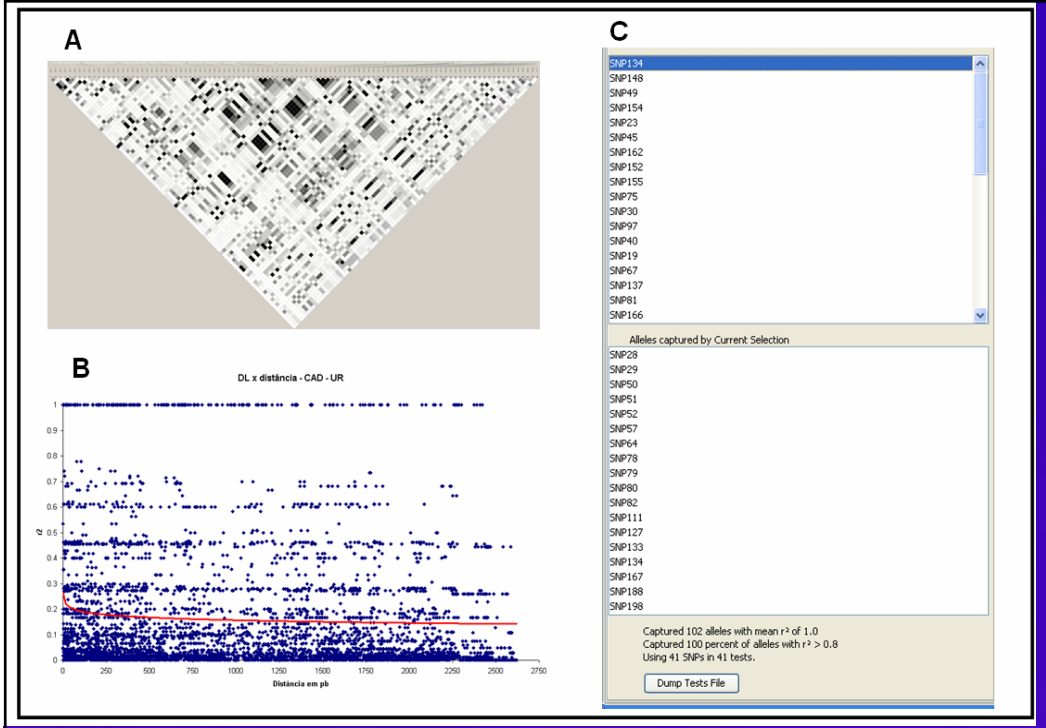
Deployment of a large number (> 1000) of F2 progeny individuals to maximize probability of generating a recombinant individual with a superior multiple QTL allele content

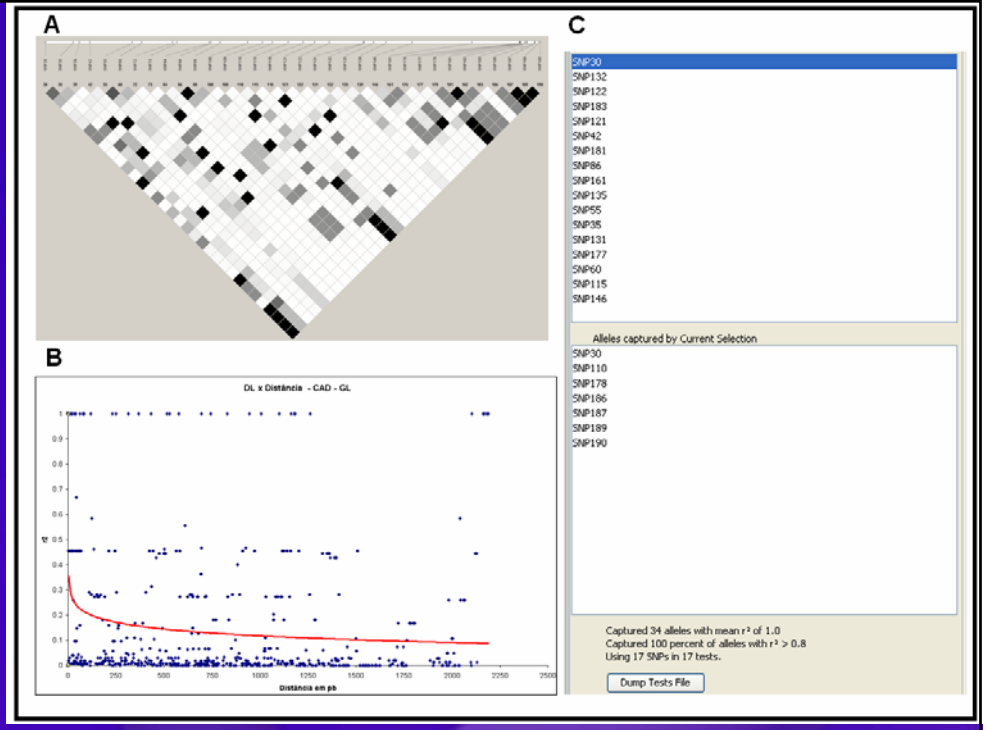


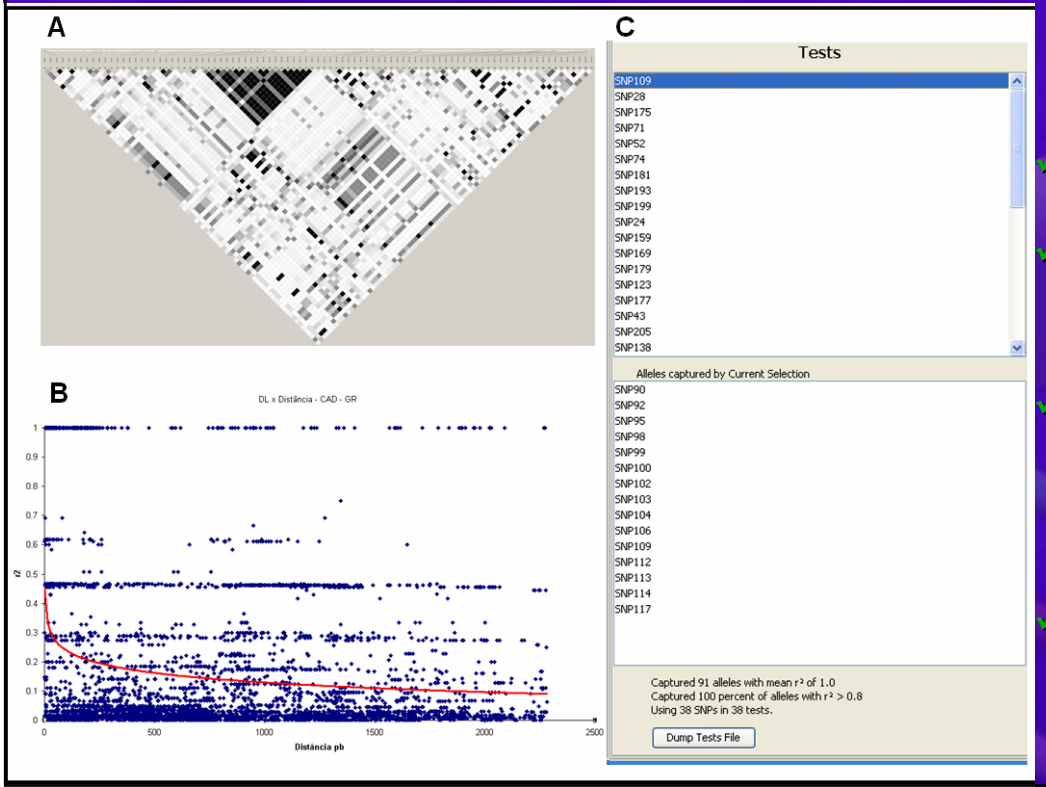
Genotyping > 1000 seedlings with a small (~ 6 to 10) set of flanking markers for a targeted number of QTL for wood quality traits

Early marker assisted selection. Selection intensity is increased by MAS for late expressing traits but number of trees commonly deployed in progeny (~100) test is kept the same, thus allowing large variation to select for other traits such as volume growth, form and branching habit.


QTL
QTL or candidate gene
Gene and exact polymorphism (QTN) identified
- Resolution +
LE markers – Linkage Equilibrium
Ex. Microsatellite markers flanking a QTL mapped in a high LD pedigree - Centimorgan resolution, ~ 10^5 a 10^7 bp
LD markers – Linkage Disequilibrium
Ex. SNP´s strongly associated with the QTL or candidate gene - Subcentimorgan resolution ~ 10^2 a 10^4 pb
Direct Markers
Ex. Causal SNPs (QTNs) of quantitative variation
Maximum resolution and identification of exact allele
These would be marker-trait associations useful across families







Linkage disequilibrium in the CAD gene in three *Eucalyptus* species

- ✓ ***LD:*** very rapid decay, < 500 bp
- ✓ ***NUCLEOTIDE DIVERSITY:*** very high, 1 SNP every 70 to 100 bp in coding regions
- ✓ ***TAG SNPs:*** very little redundancy – still lots of tagSNPs needed to cature all the haplotype variation
- ✓ ***SNP VARIATION:*** SNPs vary widely across species

Danielle Faria 2007

Association mapping approaches

- WHOLE GENOME SCAN
  - How many markers do we need?
- CANDIDATE GENE BASED
  - How good are the candidate genes?

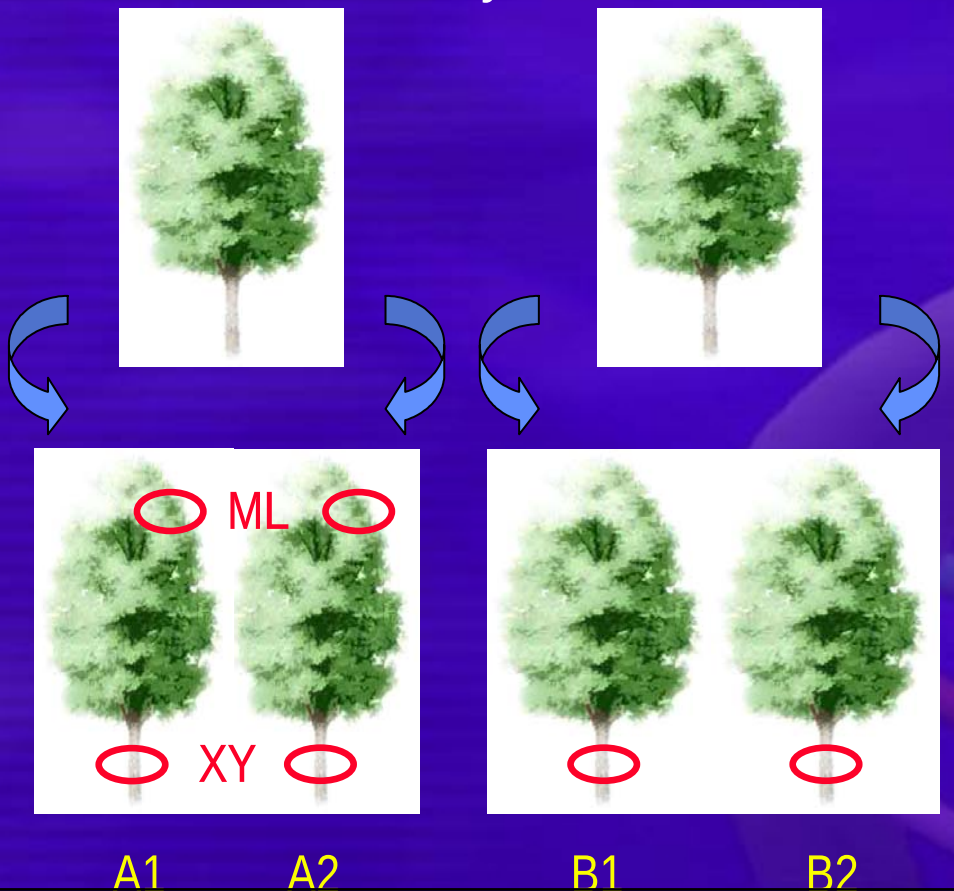
GENOLYPTUS *microarray base experiment*

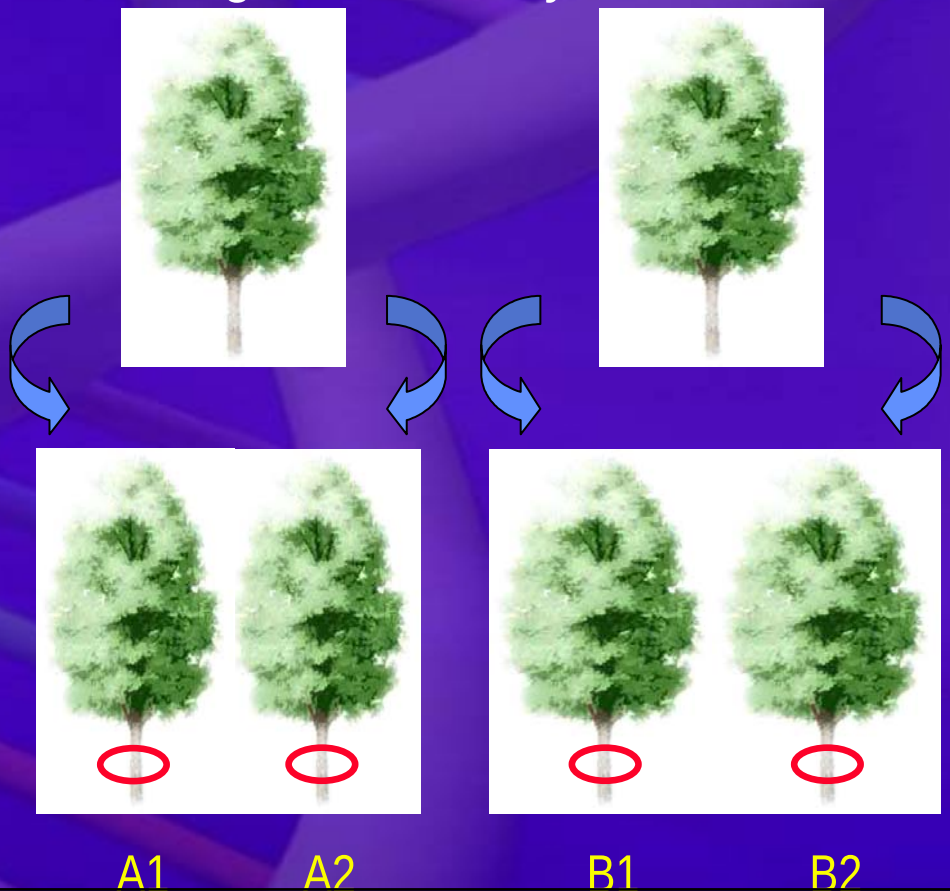
Eucalyptus grandis (GR)

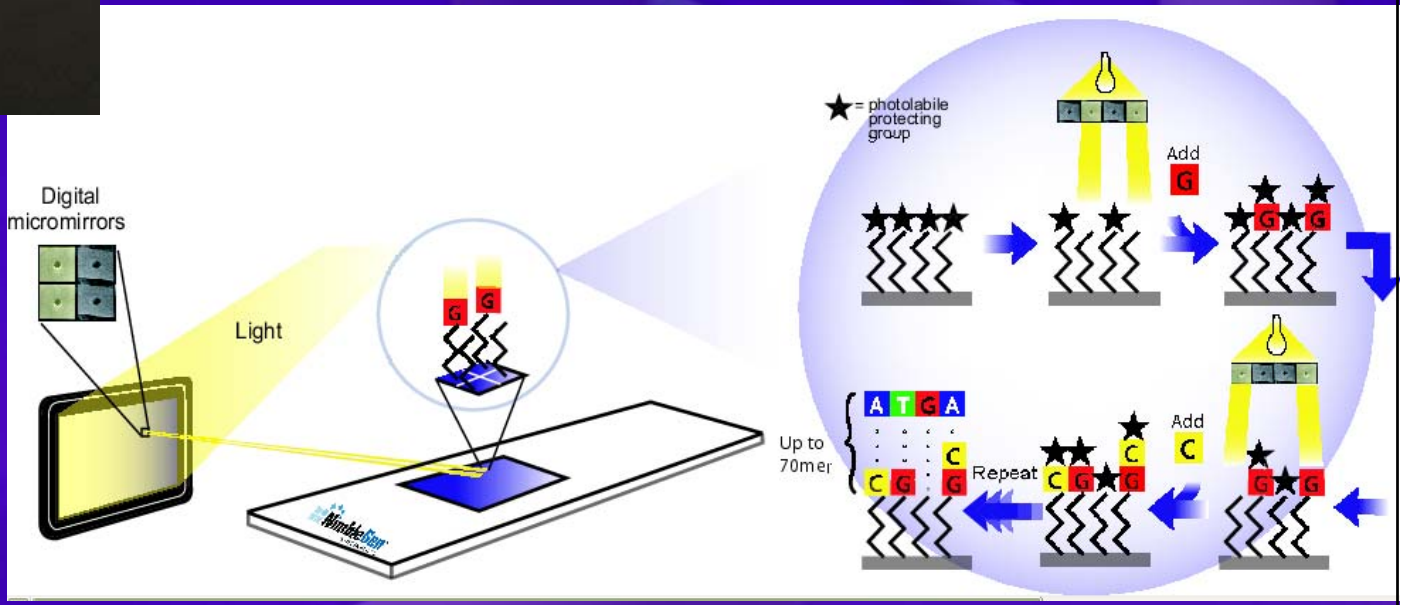
- Fast growth
- High adaptability
- High lignin
- Low cellulose yield

Eucalyptus globulus (GL)

- Slow growth
- Low adaptability in the tropics
- Low lignin
- High cellulose yield







50 n

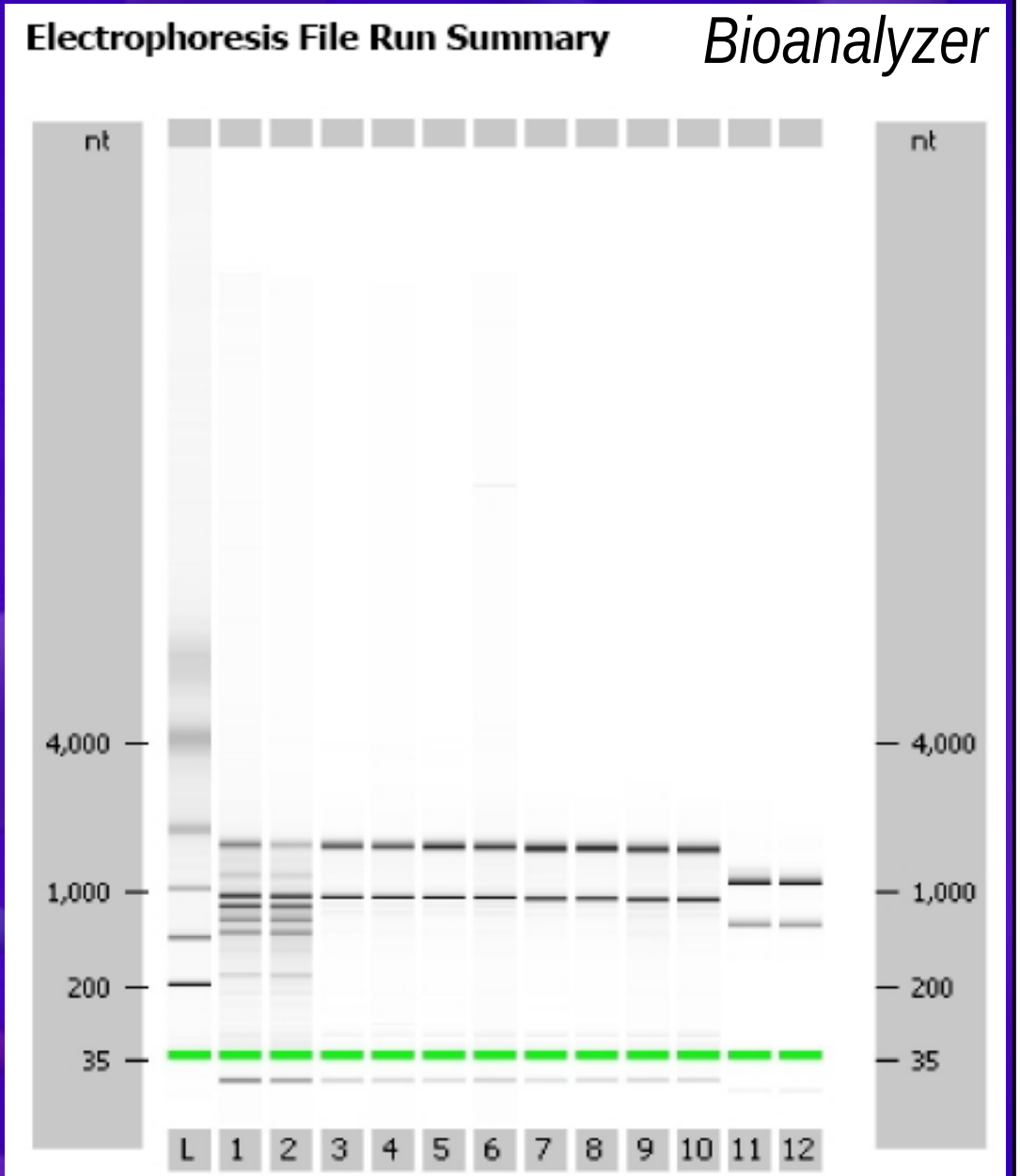
~ 800 bp EST contig

- ✓ *Each probe synthesized twice on chip*
- ✓ *Randomized distribution of probes onto the chip*

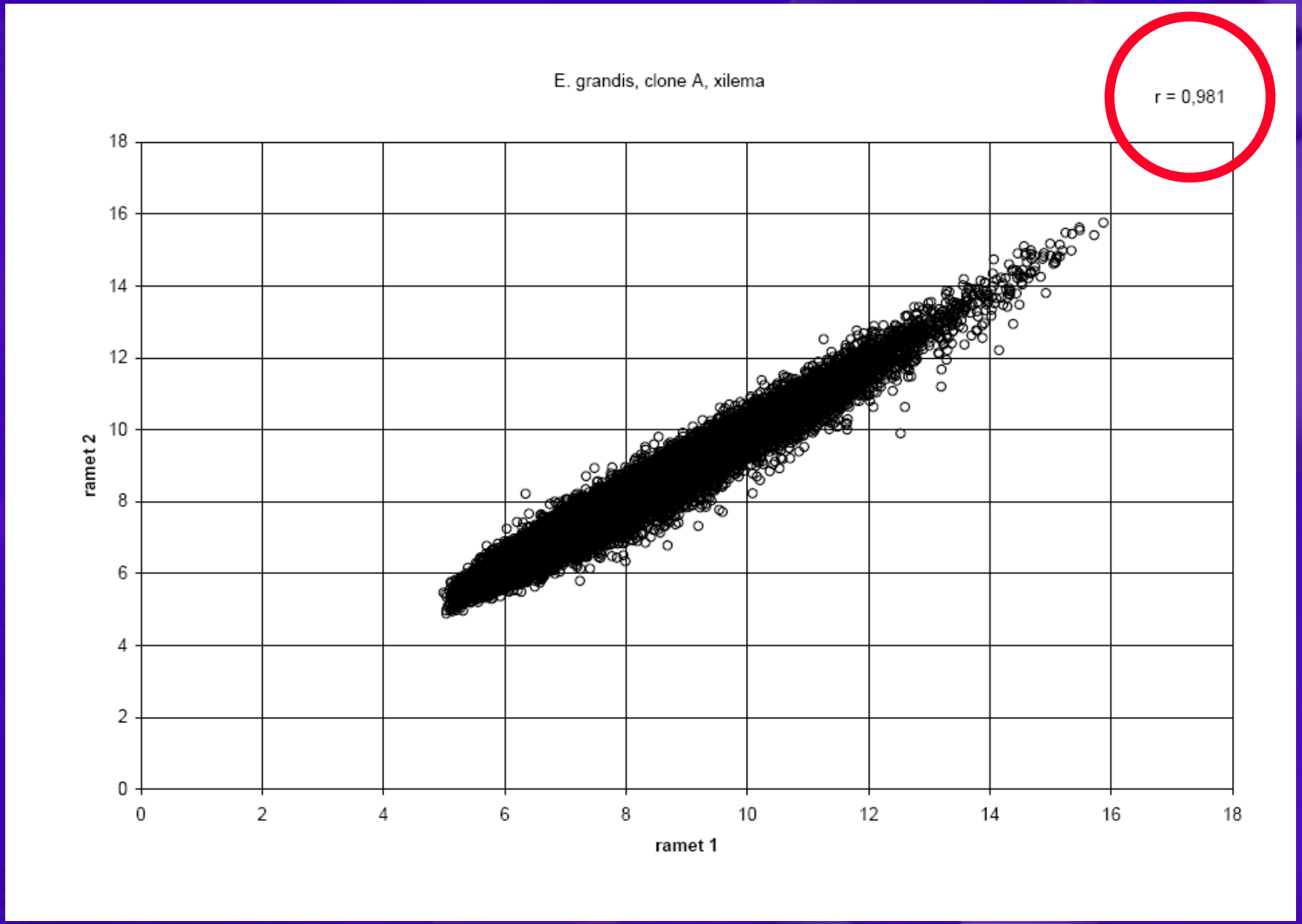
GENOLYPTUS *microarray base experiment*

NimbleGen Systems, Inc.
Reykjavik, Iceland

- *"On-chip" probe synthesis – 50mer*
- *Up to 9 probes per unigene contig*
- *Replicated probes (2X)*
- *21,442 sequences ("unigene")*
- *385,856 features per chip*
- *10 identical chips*
- *Cy3-labelled cDNA synthesis*
- *Hybridization*
- *Washing*
- *Scanning*
- *Data collection and normalization*



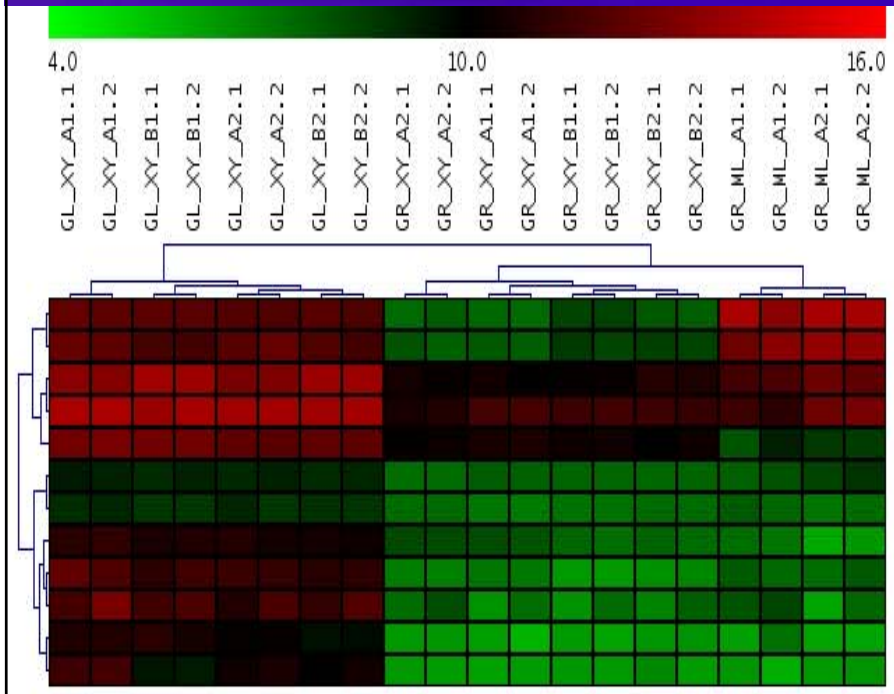
Variation between biological replicates of the same *Eucalyptus* tree



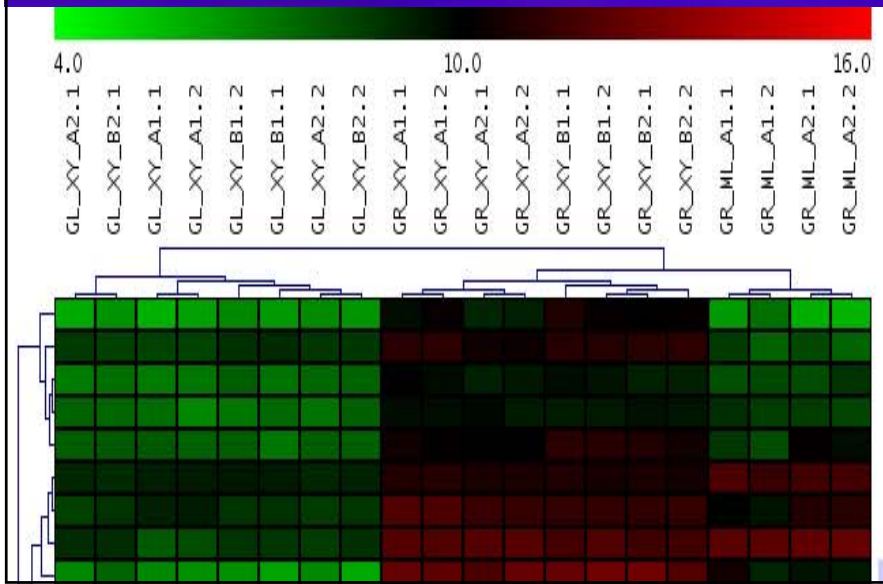
At alpha=0.01 no gene showed significant difference in expression between the two biological replicates

Xylem specific genes

Interspecific variation: *E. globulus x E. grandis*

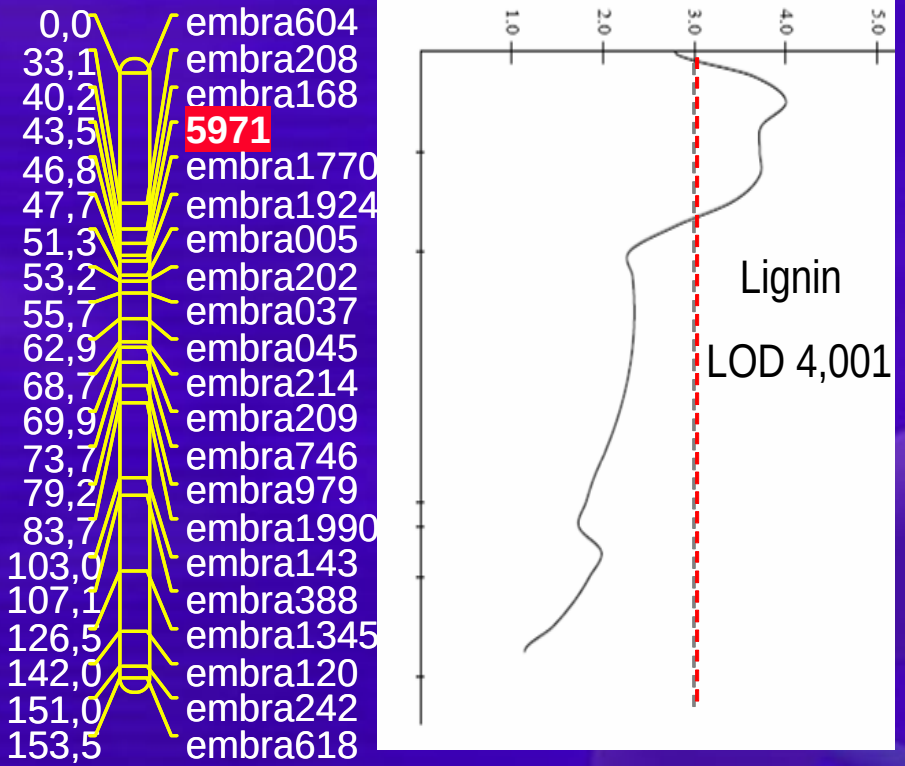


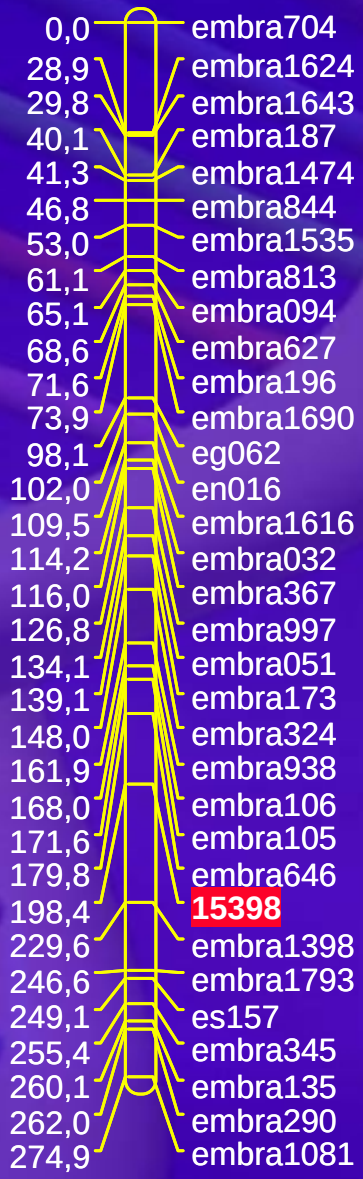
no hit
TIR-NBS disease resistance-like protein [(Populus tomentosa x P. bolleana) x P. tomentosa var. tr
unknown protein [Arabidopsis thaliana] gb|AAF79910.1| Contains similarity to SCUTL1 mRNA from Vit
putative aquaporin PIP1-2 [Vitis berlandieri x Vitis rupestris]
no hit
protein binding / ubiquitin-protein ligase/ zinc ion binding [Arabidopsis thaliana] gb|AAM19965.1
unknown protein [Arabidopsis thaliana] gb|AAM62838.1| Yippee-like protein [Arabidopsis thaliana]
unknown protein [Arabidopsis thaliana] gb|AAM97053.1| unknown protein [Arabidopsis thaliana] gb|A
Protein of unknown function DUF567 [Medicago truncatula]
no hit
no hit
SKP1 component [Medicago truncatula]

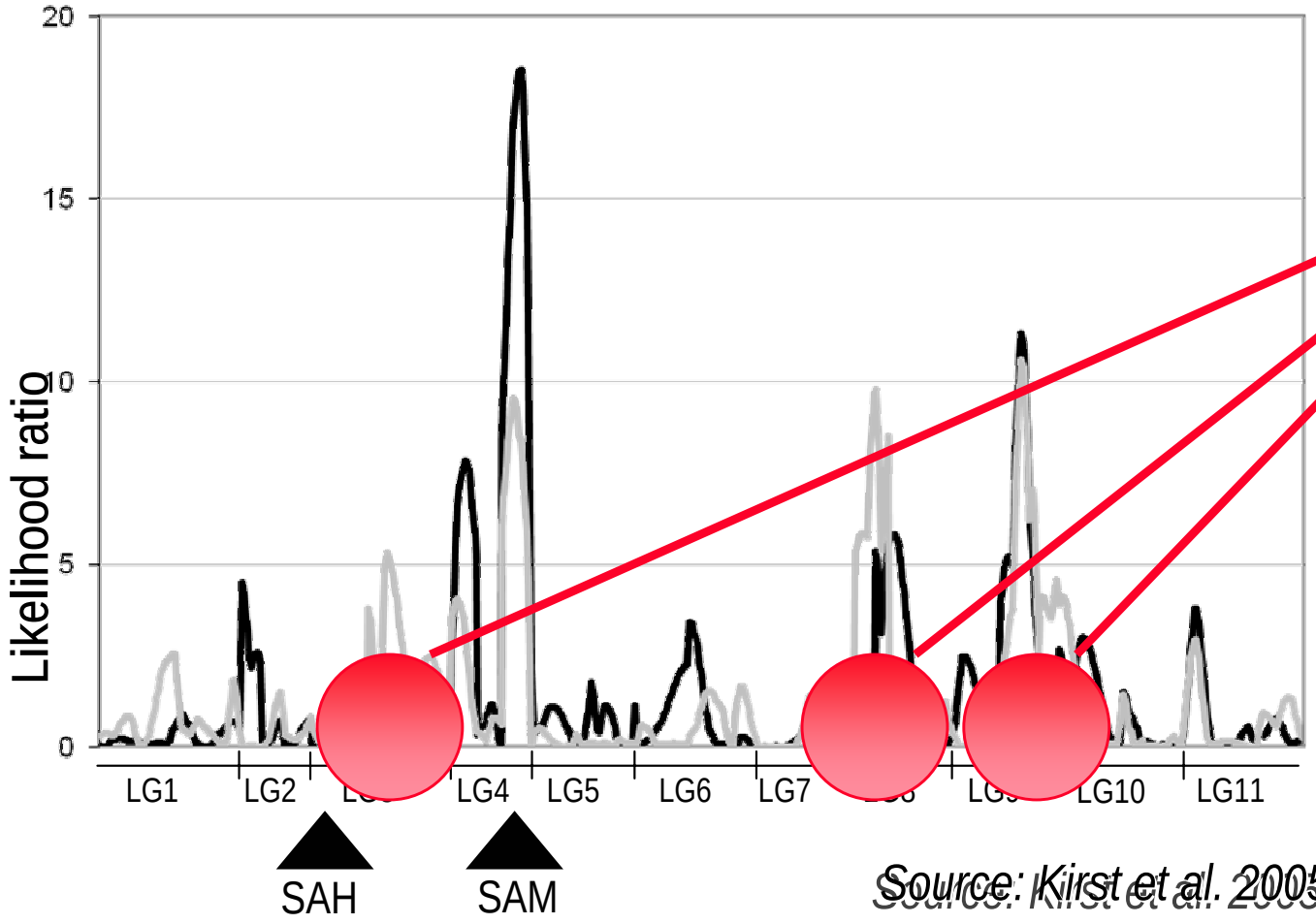


no hit
no hit
unknown protein [Arabidopsis thaliana] gb|AAD10685.1| Hypothetical protein [Arabidopsis thaliana]
alpha-mannosidase [Arabidopsis thaliana] gb|AAM47314.1| AT3g26720/MLJ15_12 [Arabidopsis thaliana]
Mal d 1-like [Malus x domestica]
nucleotide binding [Arabidopsis thaliana] gb|AAL47352.1| WD-repeat protein-like [Arabidopsis thali
Pyridoxal phosphate biosynthetic protein pdxA [Rhizobium sp. NGR234]
Similar to gb|X84260 POS5 gene product from Saccharomyces cerevisiae. EST gb|W43879 comes from th
zinc transporter [Eucalyptus grandis]

Co-location of differentially expressed genes (*E. grandis x E. globulus*) with QTLs







To what extent is variation in gene expression associated with variation in complex traits at the phenotypic level?

QTL mapping experiments are now being expanded to expression QTL mapping experiments to provide a global analysis of *cis* and *trans* eQTLs and identify candidate genes for complex traits

Single Feature Polymorphism pseudo-testcross screening

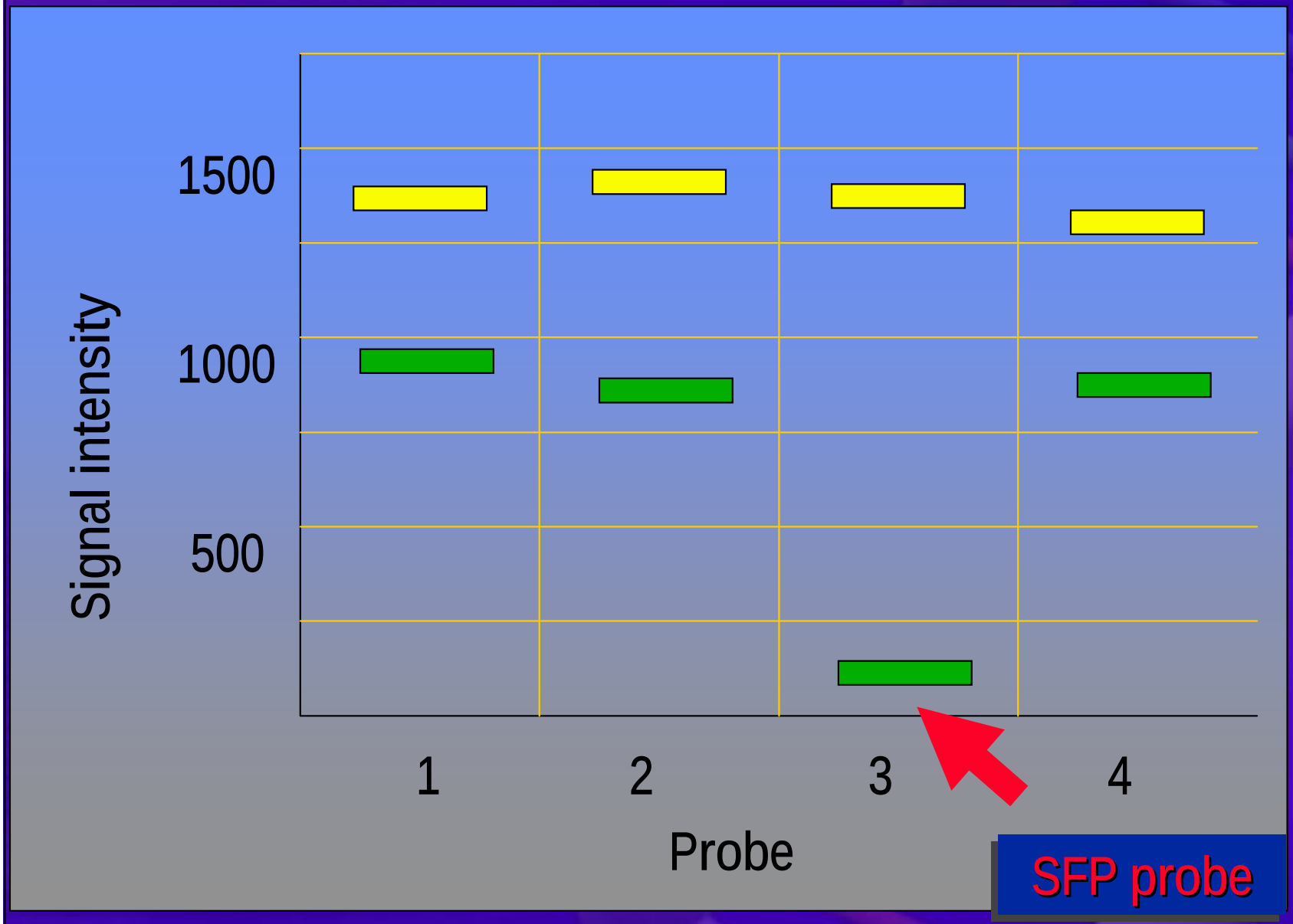
PLANT MATERIAL

- ✓ Two segregating F1 families (both have a ~250 microsat map)
  - ✓ *E. grandis x E. urophylla* = G38 x U15
  - ✓ (*E. dunnii x E. grandis*) x (*E. urophylla x E. globulus*) = DG x UGL
  - ✓ Both parents and 6 F1 progeny individuals

MICROARRAY DESIGN

- ✓ 1518 EST consensi from Genolyptus database - longer consensi
- ✓ 10 probes per EST consensus (designed by Agilent to cover consensus)
- ✓ 25-mers – higher probability of SNP and/or short indels detection
- ✓ RNA from leaves ; cDNA+ Alexa 555 ; two biological reps

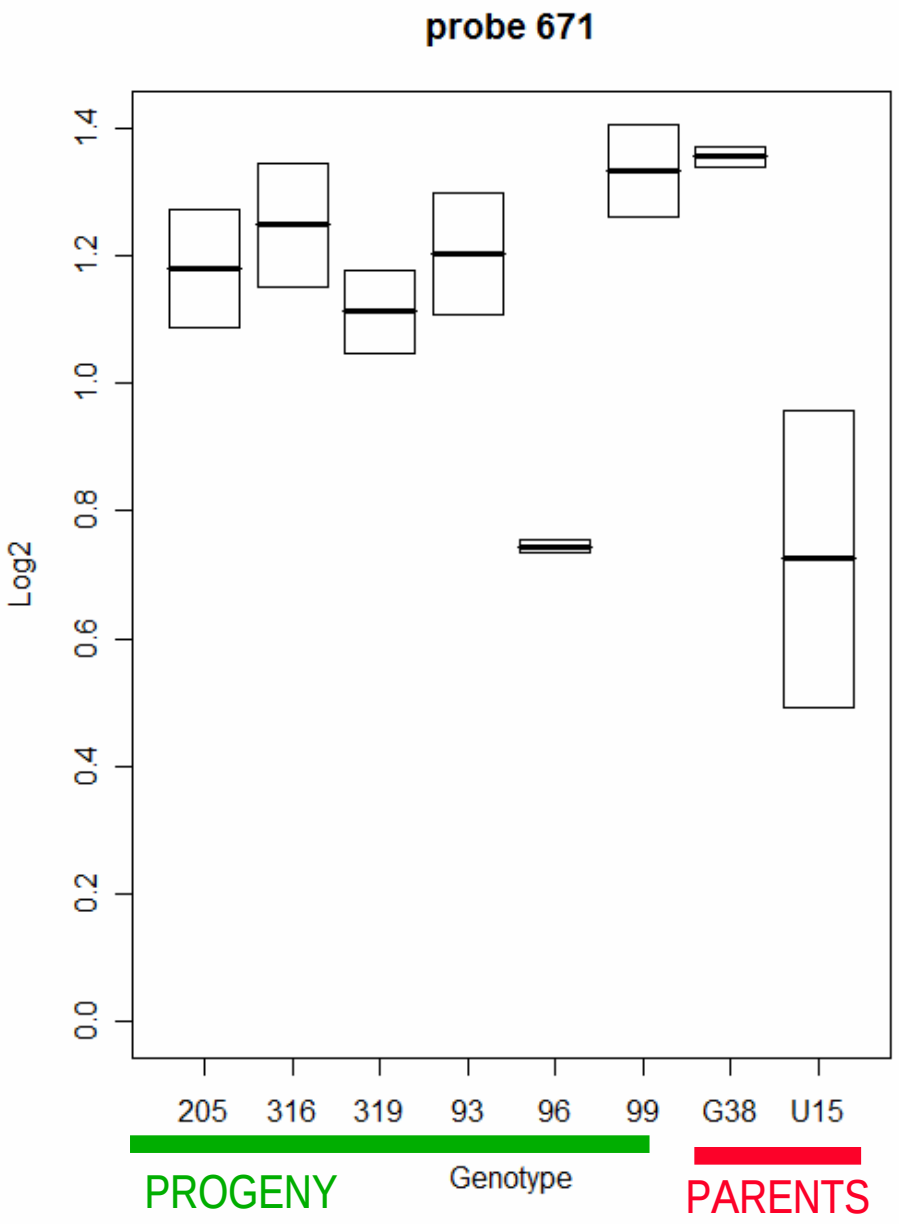
Detection of putative SFPs by analyzing *probe x genotype* interaction

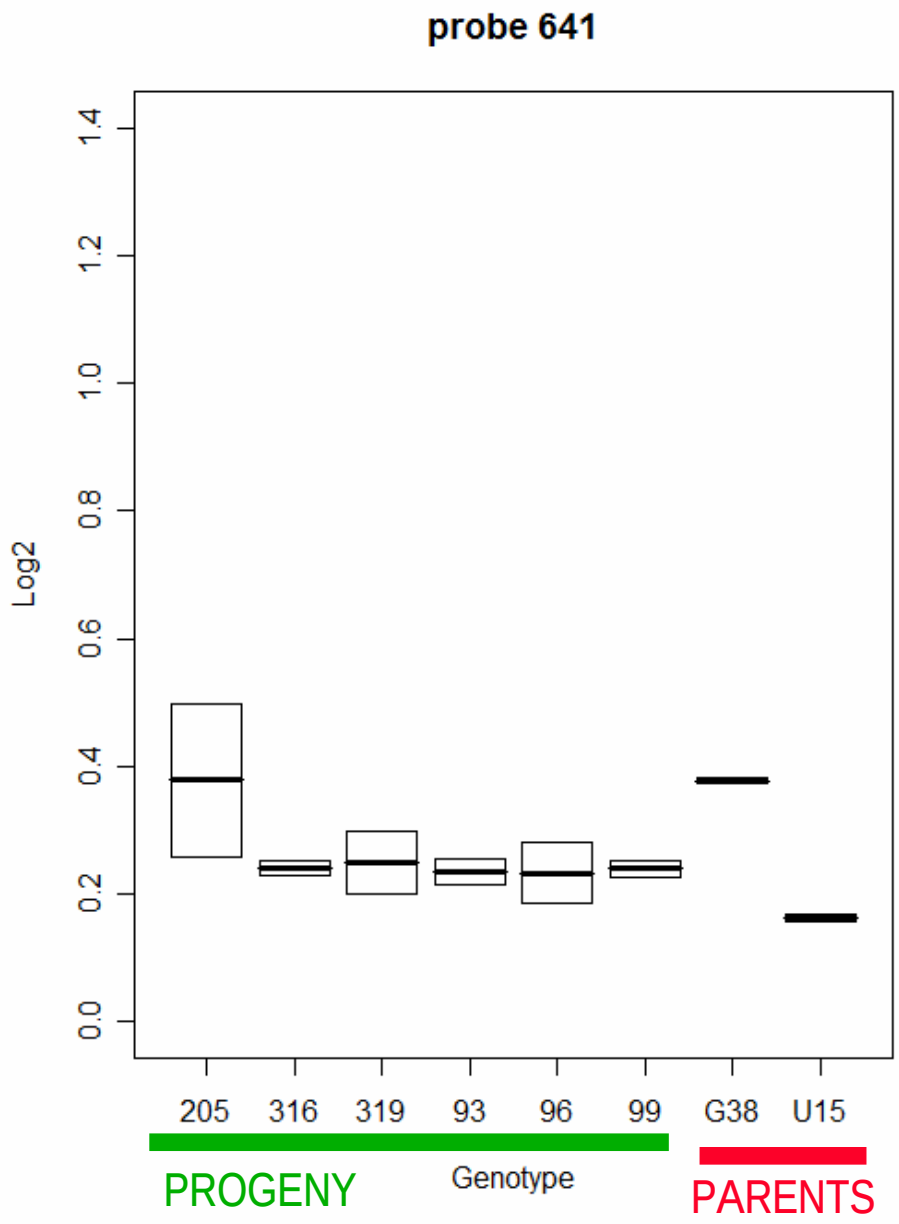


Adapted from Roald et al. 2005 Genome Res. Courtesy of Derek Drost - UFL

Significant SFP by LSM and K-means clustering

Significant SFP by K-means clustering but not by *LSM*





EFFICIENCY OF SFP FOR MAPPING GENES

- Family screening by pseudotestcross allows robust declaration of SFPs
- Using a second family almost doubles the number of mappable genes
- For the current 21,403 unigenes using a stringent statistical analysis we expect at least ~ 3500 genes to be mapped;
- Many more could be mapped if using only cluster analysis cut-off

FAMILY AND SFP SCREENING PHASE: two-step screening:

- Family screening in small array (15,000 probes)
- Probe screening in large array (200,000 probes)

- SFP GENOTYPING AND MAPPING PHASE:
  - Family specific SFP array: selected probes - small array 15,000 probes
  - Selective genoyping: ~96 more informative progeny individuals based on SSR recombination map data

Perspectives of applied genomics in *Eucalyptus* breeding

- ✓ *Genomic resources will be abundant and public*
- ✓ *No need to choose candidate genes*
- ✓ *Cost reduction of genomic methods: SNPs will be typed by simply resequencing the whole genome (today 2Gb at ~3,000 USD)*
- ✓ *Several genomes will be sequenced for association genetics studies*
- ✓ *Short reads will be mapped onto the reference sequence of E. grandis*
- ✓ *Main limitation: availability of appropriate structured material, sufficiently replicated across field sites and precisely phenotyped for traits of interest*
- ✓ *Genome-wide selection methods to capture all relevant genetic variation*
- ✓ *Perspectives of applied genomics in Eucalyptus are encouraging given the existing variation, the upcoming draft genome and the evolution of better and cheaper genotyping technologies*

Some thoughts on the use of GM *Eucalyptus*

- Technical issues
  - *Targets of modification: what genes and how to modify ?*
  - *Transformation protocols: genotype dependence, efficiency*
  - *Long term stability and tissue specificity of gene expression*
  - *Tree sterility, gene flow, propagation system*
- Economic issues
  - ***Relative gain: GM x natural variation***
  - *Intellectual property: patent licensing, freedom to operate, royalties*
  - *Biosafety : GM trial time +rotation time (harvest) x duration of patent*
  - *Who has the technology x who has the genetic material*
  - *Investiment risk: regulaiton and biosafety issues still unclear*
- Political issues, public perception, client demand
  - *Forest certification (FSC)*

HOWEVER GM TREES LIKE IN ANNUAL CROPS COULD REPRESENT A TECHNOLOGY DIVIDE AND NECESSARY CONDITION FOR A FOREST BASED INDUSTRY TO REMAIN COMPETITIVE

Shinitiro Oda

Suzano breeder who developed and selected BRASUZ1

Why did we want a more homozygous genome?

- ✓ Eucalyptus is preferentially outcrossing (~90% outcrossing rate) with late acting self incompatibility
- ✓ Heterozygosity throughout the genome is very high
- ✓ Nucleotide variation in *Eucalyptus* is also very high (1 SNP/~100 bp)
- ✓ Difficulties expected for whole-genome shotgun assembly due to high within-individual haplotype variation
- ✓ Humans: recent paper of the Venter genome developed methods to assemble the two alternaive alleles
- ✓ Ex. Grape genome project: looked for a more homozygous variety

All grapevine varieties are highly heterozygous; preliminary data showed that there was as much as 13% sequence divergence between alleles, which would hinder reliable contig assembly when a whole-genome shotgun strategy was used for sequencing. Our consortium therefore selected the grapevine PN40024 genotype for sequencing. This line, originally derived from Pinot Noir, has been bred close to full homozygosity (estimated at about 93%) by successive selfings, permitting a high-quality whole-genome shotgun assembly.

History of the target tree BRASUZ1

- 1968: Seeds from *E. grandis* Coffs Harbor (Australia) were bought by Suzano and a commercial stand was planted in 1968 in São Paulo state
- 1974: mass selected trees in this commercial stand for volume and form and collected seeds from them
- 1975: establishment of an open pollinated progeny trial with seeds
- 1979/1980: best trees selected between and within families in the progeny trial; trees cloned by grafting
- 1982: clonal seed orchard established with selected trees
- 1986: selfing program of all trees in seed orchard
- 1990: surviving S1 seedling established a seedling seed orchard composed exclusively of S1 trees among which 7D now BRASUZ1 is one of them.
- 2008: BRASUZ1 is now 18 years old. It has good general combining ability. and is resistant to *Puccinia* rust

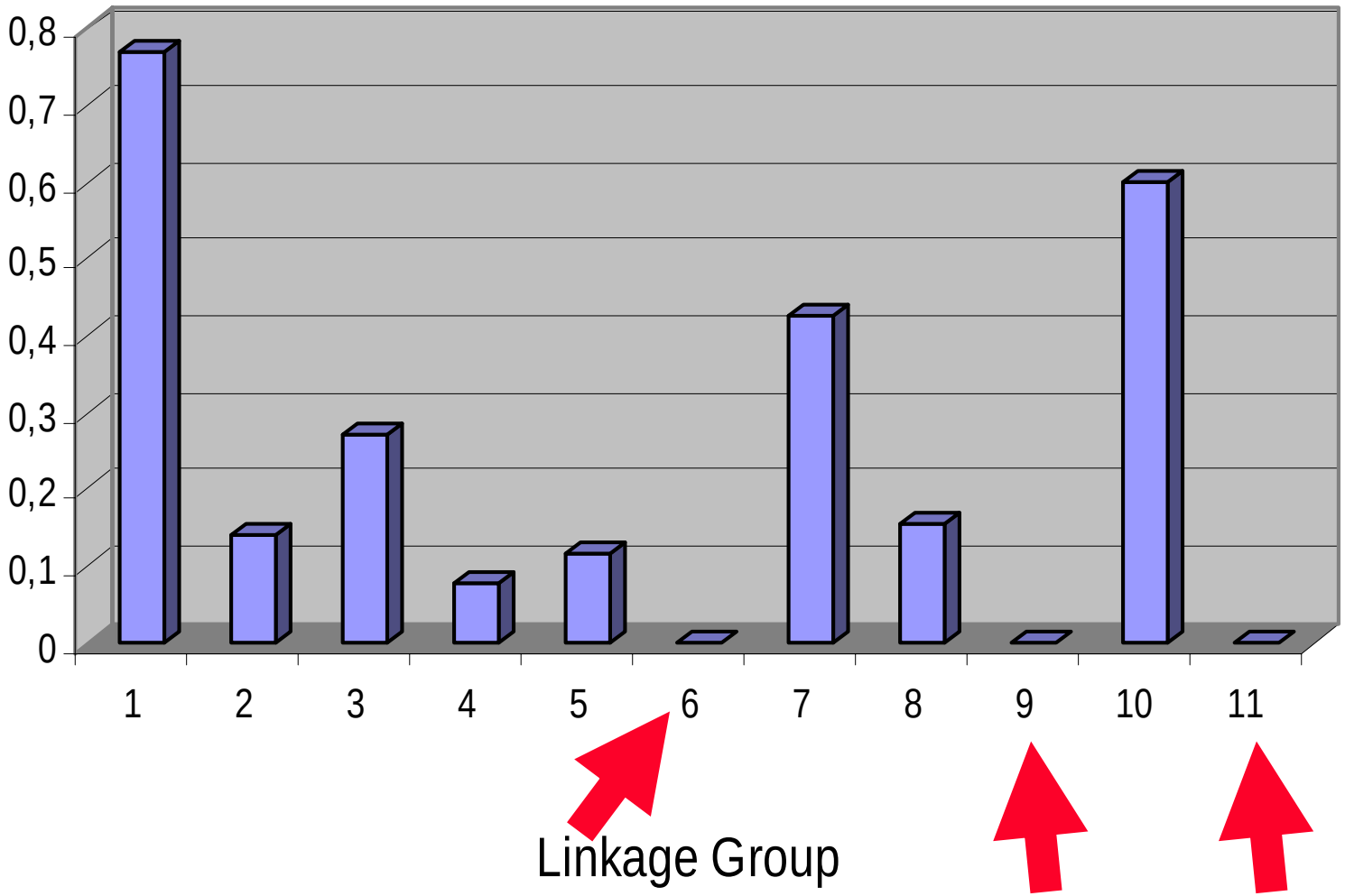
Seedling seed orchard of S1 trees where BRASUZ1 is located in Itapetininga. São Paulo State

Tissue collection for RNA:

JGI will generate a large EST collection for BRASUZ1 using combination of Sanger and 454 and Illumina

RESULTS: increase in homozygosity by linkage group

Significant variation across linkage groups suggests variable tolerance to homozygosity due to variable distribution of genetic load



BRASUZ1 increased homozygosity in relation to a regular *Eucalyptus grandis* tree was estimated at 21%

Linkage group	cM	# markers	# inf.markers	Homozygous	Heterozygous	% homozygosity	% adj.homozy.
1	76	16	13	10	3	0,77	0,06
2	117	28	21	3	18	0,14	0,02
3	54	14	11	3	8	0,27	0,02
4	60	16	13	1	12	0,08	0,01
5	110	26	17	2	15	0,12	0,01
6	115	30	11	0	11	0,00	0,00
7	63	17	14	6	8	0,43	0,03
8	77	17	13	2	11	0,15	0,01
9	60	18	13	0	13	0,00	0,00
10	72	16	10	6	4	0,60	0,05
11	98	14	11	0	11	0,00	0,00
	902	*212*	*147*	*33*	*114*	*0,23*	*0,21*

BRASUZ1 total homozygosity estimated from microsatellites:

212 microsats

65 already homozygous

33 went into homozygosity following selfing

98 microsatellites are homozygous

98/212 = 46% homozygosity


27 4 2006

Acknowledgments

- *THIS IS THE WORK OF A LOT OF PEOPLE!*
- All university and company scientists, graduate and undergraduate students that participate in the GENOLYPTUS project
- All 22 institutions that constitute the GENOLYPTUS project
- Brazilian Ministry of Science and Technology
  - FINEP – Fundo Verde Amarelo
  - CNPq