Pine Genomics Workshop

May 21-22, 2003

University of California, Davis, CA

At the invitation of Dr. David Neale, USFS and The University of California, Davis, a small group of forest geneticists and molecular biologists gathered at UC Davis for an informal workshop on the status of loblolly pine genomics research in the United States. Workshop participants represented 6 major American academic institutions, 2 USFS research stations, and 1 forest products company (Appendix 1 – participants list).

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Summary Report

Submitted by Nicholas Wheeler

Purpose of the Meeting

The purpose of the meeting was to explore opportunities for developing a cooperative research organization and improving the funding status of genomics research in loblolly pine in the United States.

Problem Statement: As described by Dr. Neale in his opening remarks, genomics research for trees in general and pines in particular is poorly organized and woefully under-funded despite the stature of forest products in the nation's agricultural economy (timber is the single highest valued crop in the USA). Currently, at least 11 annual crops and one angiosperm (poplar) enjoy greater genomics research support than pines. The pine research community has failed to organize and market their varied capabilities to the funding community. As a result, research proceeds in a disjointed and halting manner, with significant overlap and redundancy.

Proposed Solution: Explore opportunities and means to form a multi-institutional network of research collaborators with a common and well-defined set of genomics research goals. Ostensibly, such a group would be viewed by the funding communities as a credible and organized entity capable of delivering results in an efficient and rapid manner.

Meeting Approach: The meeting was functionally divided into two sessions. The first session provided a forum for participants to describe the current research status of EST discovery; gene expression; maps, populations, QTLs, Markers and SNPs; bioinformatics; and database / genetic stock archives. Session two allowed for small break-out groups to define: 1) the level of resources that currently exist, 2) specified goals and resources needed to achieve them, and 3) a five year plan outline to achieve goals. Four groups then reported out on the following topics: Gene Discovery, Functional Genomics, Maps, and Genetic Stocks / Germplasm.

Meeting Product: The product of this meeting will be a report summarizing the breakout session results, including action statements for achieving the 5 year plan. A second meeting to be held within 6-12 months is anticipated.

Purpose of this Document: To capture the essence and insights of this meeting and provide a framework for the final report.

Group Discussion Following Introduction

Prior to delivery of status updates, participants contributed a series of comments and suggestions. These are presented in order, with commentary (NW) in italics.

- Barry Goldfarb_ What do other research groups consider to be "genomic resources"? Knowing this might help us define where the holes are in the pine community. What is the perfect model organization? *To my knowledge, neither of these questions was answered, though Arabidopsis was offered at one point as a model for genetic stock centers.*
- John Cairney_ One reason why genomic funding for pines might suffer is because they are viewed as non-facile organisms. But then, so are humans. The two share a lot in common, biologically.
- Richard Michelmore_ You should look for ways to separate your crop from all others. What can be done with trees that can not be done with other species? What is unique about pines? Turn limitations into strengths. *A list of items considered unique to pines/conifers was later collated. This is noted in a section to follow.*
- Craig Echt_ How do other groups work? Do they have formal organizations? Do they have corporate, international agency, NSF support? Are other crop organizations more driven?
- Dave Neale_ Many other crops have very strong and organized grower support. Oddly, though timber products are represented by several small and a few very large companies, the level of external genomics supported research by these companies is almost negligible. *I believe this fact must be driven home to the major companies in the United States, supported by new results showing potential applications and their timelines.*
- Jeff Dean_ Poplar funding is appreciable and has benefited from international cooperation and politics. *I might add that much of what has been accomplished here is attributable to one very influential and well-place person, Dr. Jerry Tuskan. My point is that efforts focused at budget decision makers in Congress and major corporations are necessary to see movement.*
- Dave Neale_ His desire is to keep this discussion focused on loblolly pine in the United States. *There was no overt disagreement with this position, though later discussions raised the potential for increased opportunities by including international resources.*

Research Status Presentations

Content of presentations should be captured by breakout group reports. A very brief summary is noted here.

ESTs

- Matias Kirst, NCSU: Analysis of EST sequences by comparison to Arabidopsis. Matias suggested that short sequence reads lead to poor blast results and low homology estimates, possibly because they may be coding for UTR. Longer sequences (1Kb) have very high homology with Arabidopsis. Conclusions:
    - Highly expressed sequences are not more conserved.
    - Similarity between genomes not a function of conserved domains.
    - There is relatively low uniqueness in wood transcripts.
    - The same is true for pine genome in general.

- There appears to be a core transcriptome in higher plants.
- Diversity of plants is possibly due to differential expression or regulation of homologous genes.

- Jeff Dean, UGA: Described status of IFAFS root EST project (13K 3' end root ESTs sequenced). Goal is to get 5' end and ultimately complete sequence for these. Described status of new NSF grant to sequence an additional 70K (?) transcripts from stems, needles, and roots of loblolly pine libraries derived under several types of stress using 3 genotypes (clones provided by UF). Lee and Marie Pratt described the remarkable pipeline in place for bioinformatics of Georgia data. ***A comment was made by Richard Michelmore that efforts must be made to integrate data across EST projects! A central repository is needed***.

- John Cairney, IPST: Described status of his project entitled "Gene expression during embryogenesis in loblolly pine". Listed objectives including construction of 9 libraries from somatic and zygotic embryos and megagametophytes; construction of 200 and 10,000 element microarrays; and a series of probes of arrays. Ultimate goal would be to have full-length sequence.

Gene Expression

- Gary Peter, IPST/UF: Gary reported on status of collaborative NSF grant held with Len van Zyl (NCSU). The project relies on gene expression studies using a 2300 EST microarray using modified Klenow methods that yield excellent signal to noise ratios. Probe experiments include comparisons of early wood and late wood, normal vs compression wood, and somatic embryos from spruce and/or loblolly pine. Discussion was held on cost of 70mer oligo sets: current estimates are $30/70mer, or around $500K for a 20,000 element array. Functional genomics needs in pine were listed as 1) a unigene oligo based array, 2) proteomics capabilities, 3) genetic transformation, 4) plant materials / clones available to the research community, and 5) centralized database capability.
- John Davis, UF: John described efforts to study gene expression following challenge with pathogens using membrane arrays. This is basically a study of signal pathway architecture. Functional analysis is also being pursued through transformation in heterologous systems (tobacco, Arabidopsis). He described the collaborative association genetics studies and the clonal materials in use by the ADEPT team. Future needs were defined as 1) access to good germplasm, 2) transformation systems for loblolly pine, 3) centralization of expression arrays, and 4) physical maps of pine.

Maps, Populations, QTLs, Markers and SNPs

- David Neale, UC Davis/USFS: Provided a description of two archived loblolly pine mapping populations (IFGBAS and IFGQTL), both of which are large, 3 generation full sib crosses. These populations are available to the public for

placement of markers or QTLs. A consensus map of the two, with over 300 markers (ESTs, RFLPs, isozymes) exists. A reference population of 96 individuals for each cross is archived on Weyerhaeuser land. Dave also described 3 association populations, either in existence or under construction. Finally, reference was made to comparative mapping efforts in the Pinaceace, with loblolly, Radiata, slash, maritime and Scots pines currently being tied together. The message from this work is that it appears the genomes of the over 100 species of pine are very similar, and may be viewed as a single genetic entity. *This is a unique feature.*

- Craig Echt, USFS, Mississippi: This presentation described the lab infrastructure and molecular marker capability. They are a large volume lab working predominantly with SSR and RAPD markers, but hoping to grow in SNP capability. The staff will grow to include cytogenetics capability (physical mapping?). They are currently placing SSR on the IFGBASE and QTL maps, as well as genotyping the WeyCo association population with 36 SSR markers in search of population structure issues that may interfere with interpretation of association results. They hope to eventually have 120 – 200 publicly available SSRs on the loblolly map.
- Barry Goldfarb, NCSU: Barry describe 3 clonal pine populations of potential value for genomics studies:
    - Seedling/cutting trial est. 1990/91 with 9 fs families and 50 clones. This population is currently under study for wood chemistry, physical wood properties, disease, growth, metabolic profiling and microarray work.
    - Clonal selection study est. 1998 with 8 unrelated crosses and 450 clones, un-replicated, on 2 sites. Currently evaluated for growth and rust. Over 400 clones still in hedges, but about to be destroyed.
    - Association mapping population, started in 2003, with 500 unrelated clones targeted. Population was a component of an unsuccessful grant to NSF, but still in development.

  Barry noted the needs of the future include having appropriate genetic material for cloning, good cloning tools (SE, rooting), and good lead time to develop populations. SE required to match up with transformation studies. He noted the precarious nature of his rooting program.

Bioinformatics

- Lee and Marie Pratt, UGA: Web-based presentation of information pipeline for dealing with EST sequences, describing capabilities and flexibility of bioinformatics tools developed at UGA. *The attention given by this group and the full-service capability displayed suggest this unit would be an excellent choice for centralizing loblolly pine genomics databases.*

Database and Genetic Stocks

- David Neale, UC Davis and USFS: Dave briefly described the curation function of the Dendrome server, noting that it has been maintained for several years without funding. This service to the community is critical but historically under-funded in most crops. Problems with database entries occur because of inconsistent formatting of maps etc in the literature. Unlike sequence data, journals seem to have no conformity standards for other types of genomic information. Stock center needs include a repository need for things like cDNA clones, primer sets, activation tagging lines, mapping populations, microarrays, sage tags, BAC libraries, etc. Models for this include the Maize Center and the Arabidopsis Center. NSF is an obvious source of secured funding for this effort.

- Tom Byram, Texas A&M: Closing comments on germplasm were made by the Director of the Western Gulf Tree Improvement Program. He noted that his coop is made up of 9 corporations and 5 state agencies but no Universities or Federal membership (where much of this genomics work is taking place). Thus, ownership of plant materials is widely distributed with a great range in willingness to share for R&D purposes. Legacy tissues and tests are rapidly disappearing and the program matures. While the 1st generation orchards were large and diverse, the later orchards are much less diverse and geographically represented. Some clone banks exist, but not all material is preserved. There is a move to small, elite populations and the use of complementary breeding designs that may yield large, full-sib families useful for QTL mapping. Efforts to share materials for research require time and the uses of codes. *One of the unique features of working with conifers, the highly diverse genetic resource, may be jeopardized slightly, although remaining materials are still highly heterogeneous and heterozygous.*

Open Discussion Following Presentations

The participants were challenged with the question "Do we need a Pine Genome Project?". Responses generally deviated from the request to raise other questions and issues. These responses are paraphrased as noted below:

John Davis: I think it is a great idea.

Craig Echt: Why have a project? What is the goal?
Dave response: To move pine out of the funding basement.

Lee Pratt: Believes that the first requirement of such a project would be the creation of a physical map of pine.

Dave response: Certainly that could be a goal of the project, with a need for large insert libraries.

Craig Echt: Again, driving at the need for a goal: We need a vision for moving up the ladder and getting more money. What are the drivers for doing this?

Dave: The tree improvement model. More fiber, less time, lower costs. To do science better.
Gary Peter: Conservation of land-base through greater efficiency of fiber production.
Barry G: We still have natural systems to work with.

John Cairney: We need to evaluate a number of issues relative to the creation of a project.

What is the perfect genomics project model?
What restrictions or limitations does the pine genome have?
What unique features exist in the pine genome/species to make it attractive for funding?
Is there a true willingness to get it done in this room?
We must consider international issues.

The group grasped upon the third of these questions to itemize potential unique features of the crop, pine.

- Woody, perennial habit
- Lives to great age (*genotypes can be retained for long periods*)
- Gymnosperms – ancient plant lineages.
- Basically possesses an undomesticated genome
- Ecologically dominant organisms
- Reproductive biology, including the haploid megagametophyte and ability to do SE.
- Can be clonally propagated.
- Single genetic entity (all 100 species share genome).
- Trees as ecosystems
- Large, stable genome.
- Not typically susceptible to viruses.

At this point it was suggested (Tim Mullin) to refocus the group efforts and return to defining the vision and needs of the group. Participants were asked to identify potential goals or targets for a project, which would be appealing to the funding community.

Jeff Dean: To develop a complete genome unigene set of oligos as diagnostic tools to evaluate tree phenotypes and genotypes and provide predictive values.
Jeff also offered: To develop the capability of precise molecular manipulation through transformation and knock-out technologies.

Edgar Fuchs: Goals can be categorized into scientific, societal, and economic. Dave added that an economic goal would be to develop MAS/MAB capabilities, and a societal goal would be to insure forest health, fight climate warming (apple pie, etc.).

John Cairney: The goal would be to provide a molecular description of tree growth from embryo to maturity. He would desire development of an insertional mutagenesis technology.

Craig Echt: To address the question of why pines are so successful evolutionarily.

Gary Peter: To accelerate the domestication of pine.

John Davis: To provide a basis for understanding plant genome evolution.

Tim Mullin: To do better science through better synergy and elimination of redundancy.

The first day was brought to a close, and plans were made to break into working groups for the following session. The groups were chosen as follows:

- Gene Discovery: Marie Pratt, Alison Morse, Gary Peter, and Jeff Dean
- Functional Genomics: Matias Kirst, Gotche Kayihan, John Cairney, Lee Pratt
- Maps etc.: Craig Echt, David Neale, Nick Wheeler, John Davis
- Genetic Stocks/ Germplasm: Barry Goldfarb, Tim Mullin, Joe Nairn, Brian Baltunis

The teams were directed to consider the following topics:

- What genomic resources currently exist in your topical area?
- What genomic resources are required to meet perceived needs?
- Outline a 5 year plan to deliver genomic resources.

Each team delivered their results in a 15-20 presentation, with modest discussion.

Wrap-up

The decision was made to produce a document that summarized the findings of the meeting, and to post it on the Dendrome web site. Each group was asked to document the results of their breakout session discussions and to submit them to UC Davis for collation and report construction. To the above noted bulleted topics for inclusion in the report was added the request for defining priority activities or specific action items to achieve the 5 year plan. Participants were encouraged to talk up the attempts of this group to organize to their colleagues at home and abroad.

The schedule for document preparation was as follows:

Draft report for each breakout session completed and submitted to UC Davis by 7/1/03 (*We came close!)*

Route draft report to participants and interested parties not able to attend by 8/1/03 (*We failed miserably)*

Release to web site by 9/1/03 (*This should be up by mid December*)

Reassemble to develop action plans by _____.

Gene Discovery Subcommittee Report

Submitted by Jeff Dean
Team: Gary Peter, Alison Morse, Marie Pratt

Organismal Considerations

- Genome sizes for currently characterized pines preclude complete genome sequencing in the near future, thus genomic studies will likely focus on expressed portions of the genome.
- Some merit was seen in sub-genome sequencing efforts using either Cot or methylation-based strategies, but probably need some fundamental work repeated to verify degree of genomic repetitiveness and/or methylation. Pursuit of any such project should be closely coordinated with ongoing EST discovery programs.
- Cross-project comparisons would be greatly facilitated if one reference (type) genome were accepted by the community. Desirable characteristics in such a reference genotype might include:
    - Unfettered public access and use of the genotype
    - Facile vegetative propagation for wide distribution (at reasonable cost of access)
    - Broad representation in current breeding populations

  Representatives from major US loblolly pine breeding cooperatives suggested such trees might be identifiable from among "abandoned" founders within their programs.

Expressed Sequence Tags (ESTs)

- Current tally of loblolly pine ESTs in the public domain is approximately 75,000. Ongoing projects should yield a total of at least 250,000 public domain ESTs by the end of 2005.
- Depending on transcriptome complexity and whether or not subgenomic sequencing is undertaken, substantially more EST discovery work will be needed beyond current targets.
- Perceived high degree of conservation between pine genomes suggests that compilations of EST data across species will be useful. Some preliminary discussions have been held with Arborgen on the subject of accessing their radiata pine EST database.
- ***Note from D. Neale: There is a possibility that JGI maybe willing to sequence up to 500K ESTs in conifers in the very near term. Considerable thought regarding source materials for such an effort is required.***

Microarrays

- A loblolly pine cDNA microarray of ca. 2200 elements, based on the NCSU effort, is in use, but availability was deemed uncertain.
- A new cDNA microarray of ca. 6000 elements is in the works at UGA with plans for wide distribution to the user community at a "reasonable" cost per chip.

(Current target price of $100 per chip.) Current targets are to be in a position to ship such chips in first quarter 2004.

- Mid-range plans to regularly expand the cDNA array set based on updated "Uniscript" sets from the current EST discovery projects.
- Long-term goal is to develop microarrays based on unique long oligonucleotide (60- or 70-mer) sets from the complete Uniscript set.

Miscellaneous

- Concern was voiced over the utility of the EST sequences available from wood-forming tissues. It was noted that many of the sequences were short, and that the representative clones were unavailable. It may become necessary to repeat some sampling of these tissues in future EST work.
- Concerns were voiced over long-term archiving and distribution of clones and arrays.
- Agreement that there is a continuing need for the community to meet at regular intervals to reassess directions for the field. This would also serve to stimulate more cohesion within the community.

Recommendations

1. Encourage future meetings of a pine genomics community.
2. Identify reference genotype for loblolly pine.
3. Support continued efforts at EST discovery.
4. Establish communal resource center for producing and distributing microarrays.
5. Continue efforts to secure release of, or at least access to, privately held EST data.
6. Initiate preliminary efforts to determine feasibility of different whole genome sequencing strategies.

Functional Genomics Subcommittee Report

Submitted by: John Cairney
Team: Lee Pratt, Matias Kirst, Gotche Kayihan

The Goal of this works is to discover the role to genes/protein;

Tools That Are (Or Could Be) Used For Loblolly Pine:

Proof of function: can be demonstrated by approaches such as;

- Loss or Gain of function – overexpression of cDNA, RNAi, or antisense expression in transgenic plants
- Complementation of mutations - in Arabidopsis, Tobacco, yeast, E.coli
- Proof of function by gene transfer:
    - transfer into some conifers e.g Norway spruce, *Pinus radiata*
    - transfer into heterologous systems such as –Arabidopsis, Tobacco, yeast, E.coli
- Correlating Gene Expression with Growth Condition or developmental stage – assayed by DNA arrays, oligo arrays, differential display etc.
- Unique probes for specific members of gene families can be used to follow gene expression ---. Northerns, in-situ RNA, RT PCR, real-time PCR

Correlating Gene Expression with Phenotype

- Relate gene Expression to Maps, QTLs
- Generate & characterize mutations – assay gene expression in these mutants.
- Isolate genes and cDNA e.g. from these mutants, transform these into plants and see if mutant phenotype can be reconstructed.
    - In Arabidopsis -T-DNA or Ac/Ds transposon mutation systems have been used to generate mutations, similarly point mutants, MAP-based cloning yields genes of interest.
- 2nd site mutations can be generated that affect expression of reporter genes

Needs For Loblolly Pine Functional Genomics

- One reliable, transformable genotype of loblolly pine
  - This must be
    - freely available
    - be distributed with protocols

- Tissue specific promoters.
- Developmental specific promoters
- Stress-induced promoters
- Biotic-induced promoters
- Abiotic-induced promoters

These would allow genes to be expressed in precise ways thus could be employed to generate phenotypes in transgenic plants. Compare Expression vs phenotypes

- A large set of unique cDNAs

There is a need to centralize sequence data e.g. forward to Lee Pratt at UGA
The community needs cDNA libraries of as many tissues as possible
Suggestion: collaborate every group in US (world?) working on loblolly Pine (other Pine?) makes a cDNA library from their tissue of interest
Modified suggestion: since quality of library is important, get groups to contribute tissue and, at a few locations where workers are familiar with techniques, libraries will be made,

- T- DNA insertion, Ac/Ds insertion

Using the same approach as has been used to Arabidopsis screen for mutations
Technique could be used to screen early-expressed genes in tissue culture or somatic embryos. characterized mutants relate to mutation at single locus. Put these mutants on Fast track to array analysis
Such a program would need a large number of transformations (see problem above!)

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5 Year Plan

Transformation of Loblolly Pine: A loblolly pine genotype that is amenable to transformation should be identified. The need for a simple transformation procedure for Loblolly Pine is now so pressing that widespread collaboration is needed.

- Action Item: All Labs engaged in Loblolly Pine transformation should exchange protocols. Hold regular conference calls between active Labs
- Action needed: A coordinator is needed to set the ball rolling - to distribute e-mail addresses and phone numbers and set up the first conference call.

Isolation of Gene Promoters from Genes induced under a variety of conditions:
Not discussed in group but this is a fruitful area for inter-lab collaboration.

Unique set of cDNA's from many tissues

- Action item: transfer sequences to central location (eg Lee Pratt's lab) this lab will analyze/sort sequence and post on their website
- Action needed: Agreement by a person to act as curator. Publication of web address and protocol and format for submitting sequences,

- Action item: -generate libraries from as many tissues as possible and have them sequenced.
    - Contact labs, ask for tissue and details of genotype, growth conditions, and all pertinent information
    - Tissue will be sent to specific lab(s) that will make cDNA libraries and sequence them.

- Action needed: A Co-ordinator will be needed (John Cairney volunteers)

Will require money - write grant; the authors will be working on behalf of the community if we have a group identity and title such as the 'Pine Genomics Project /Collaboration ' then a proposal, supported by many groups, possibly with many names on the author list, could be viewed favorably by NSF.

A lab willing to act as the sequencing center must be identified and approached. Libraries could be constructed there or at other approved locations. Protocols for library construction can be distributed.

Pine labs must be approached and tissue requested

An initial Mini-project could be undertaken where we process samples from Pine Labs (ideally those that do no molecular biology, and who are thus unlikely to participate in genomics projects in the normal course of things) This could show that the system would work-especially if a number of novel sequences are generated in the process.

Arrays analysis of all tissues – large scale project. Again, if undertaken under the name of an umbrella organization, this could be funded.

Previous project (generating unique cDNAs from many sources) would provide the infrastructure for an array project.

Maps – Subcommittee Report

Submitted by: Nicholas Wheeler
Team: David Neale, John Davis, Craig Echt

This group focused on loblolly pine genomic resources related to maps and associated components such as markers and QTLs. Maps were subdivided into two major groups, physical and genetic, the latter being further subdivided into categories to facilitate discussion. These categories include: 1) Marker, 2) Phenotype (QTL), 3) Gene (EST), and 4) Fine structure.

Genetic Maps – Markers/Phenotype

Existing Resources: These two categories (markers and phenotypes) are generally so interwoven in the literature, they are discussed together here. To date, one or more genetic maps exist for 6 pine species: *Pinus taeda* (loblolly pine), *P. radiata* (radiata Pine) *P. pinaster* (maritime pine), *P. palustris* (longleaf pine), *P. elliottii* (slash pine), *and P. sylvestris* (Scots pine; Table 1). Genetic maps also exist for other members of the Pinaceae, *Pseudotsuga menziesii* (Douglas-fir) and *Picea abies* (Norway spruce, as well as a member of the Taxodiaceae, *Cryptomeria japonica* (Table 1). All together, over 70 genetic maps have been submitted to the public database housed at the Institute of Forest Genetics, USFS, Davis, CA. (http://dendrome.ucdavis.edu/). Still others are held in private hands, not currently accessible to the public.

Most of these maps have low marker density, ranging from 75 to a few hundred or more markers. RAPD and AFLP marker maps predominate in the literature, but RFLP maps for loblolly pine and Douglas-fir exist. Notably, comparative genetic maps have now been constructed between radiata, loblolly, slash, maritime and scots pines, and between loblolly pine, Douglas-fir and Norway spruce (Krutovskii et al. 2004) using a few framework RFLP and gene-based markers. These comparative studies have demonstrated a remarkable conservation of marker order across many linkage groups, suggesting the entire *Pinus* or even Pinaceace genome may be viewed as a single entity for research purposes.

Two reference loblolly pine mapping populations (IFGBAS and IFGQTL) have been immortalized in clone banks and DNA preps, and are available to users for mapping additional markers in the pine genome (http://dendrome.ucdavis.edu/). Currently, these maps consist of 139 and 238 markers, respectively; a consensus map between the two has 310 markers, split roughly as 56 % RFLP markers, 43% EST gene markers and 1% isozymes. Funding proposals have been submitted to add 250 SSR loci to these maps.

QTL maps exist for all of the species noted above. Traits mapped include growth, physical and chemical wood properties, adaptive traits, branching habits, etc. Table 1). On the whole, QTL mapping populations in trees have been undersized. Results of these studies can be expected to yield misleading information on the number of QTLs (underestimated) and the size of their effect on the phenotype (overestimated).

Regardless, the body of this research has provided for the genetic dissection of numerous quantitatively inherited traits in conifers. In addition to genetic characterization of traits, this information is valuable for identifying positional candidate genes for association studies, as more and more gene maps are produced.

New Resources Required It was the recommendation of this sub-committee that phenotypic mapping in loblolly pine continue for traits of economic and adaptive value. The existing mapping populations are not appropriate for this. Consequently, we propose the establishment of 1 or more segregating populations for community resource use. Logically, these could reside within the Tree Improvement Cooperatives. With complementary breeding designs now planned, large full-sib families will be produced routinely. Ideally, 1 or more of these could be clonally replicated to serve as QTL mapping populations. Alternatively, the USFS Southern Station (Dana Nelson) has proposed establishment of a demonstration MBS project. Such a project may be capable of supporting the establishment of community resource populations.

Placement of an additional 100 to 200 SSR markers on the loblolly pine reference maps was encouraged. Also, use of publicly available comparative mapping markers was encouraged for all future genetic map makers in pine.

Genetic Maps – Genes

Existing Resources As ESTs (Expressed sequence tag) sequences have become increasingly available in trees, methods to detect polymorphisms within them have allowed for the mapping of specific genes to linkage groups (Temesgen et al. 2001; Cato et al. 2001). Typically these are being added to marker/QTL maps. In loblolly pine, 19 candidate genes have been genetically mapped and added to the reference map noted above (Brown et al. 2003). Gene mapping is also taking place in the EU and New Zealand (Cato et al. 2001).

SNP detection in candidate genes is now occurring at a rapid pace. The Neale lab at UC Davis has identified 286 SNPs in the 19 candidate genes noted above. SNP mapping of candidate genes will likely become increasingly common in the years ahead. SNP discovery projects in Radiata pine, Scots pine and maritime pine are also underway.

New Resources Required It was proposed by this sub-committee that a uni-gene set of 3,000 to 5,000 genes from zylem, root and disease EST libraries be placed on the reference loblolly genetic map.

Genetic Maps – Fine Structure

Existing Resources Fine structure maps do not exist for loblolly pine and are relatively rare in trees. Attempts have been made to fine structure map around disease resistance genes in sugar pine (P. lambertiana; Neale unpublished) and hybrid poplar.

Resources Required The need for community resource fine structure maps is not apparent. These are viewed as independent lab requirements on a case by case basis.

Physical Maps

Existing Resources Physical maps for loblolly pine or any other conifer do not currently exist. A very restricted (0.05) BAC library does exist for loblolly pine, however (USFS, Pacific SW Station). Prior to the Pine Genomics meeting, several of the team members were encouraged by Dr. Charles Langley to not view the prospect of genome sequencing of pine as futile. The development of phosmid libraries makes this more feasible, apparently.

Required Resources The development of a physical map of any depth was viewed as a high priority for the pine genomics community. It was proposed that we engage in a pilot project to sequence a small portion of the BAC library (say 10 clones). This was viewed as a means of providing data that could be used to build a physical mapping project strategy. Physical mapping could provide valuable insight into issues such as:

- Gene family resolution in pine
- Direct identification of individual genes such as Fr1 (Fusiform rust resistance gene).
- Candidate gene verification
- Identifying the physical basis for all comparative maps.
- Understanding the distribution of the expressed genome.

It was suggested by the sub-committee that we approach the JGI sequencing lab re sequencing of 10 BAC clones.

In a subsequent discussion with a DOE contact involved in the poplar sequencing project, the following was learned:

- JGI is not interested in piecemeal work. 10 clones would not be of interest to them.
- We might be able to make a case for having the entire .05 library done. Back of the hand figuring was that this would be about twice the size of the poplar project (assumes genome of pine is 40X larger than poplar), or 6 months to do a 6x coverage
- JGI will not do less than 6 x coverage work due to the nature of random, shotgun sequencing and likelihood and coalescing
- It is worth a try and requires an initial 5 page request and proposal to one person
- The poplar people are willing to share how they proceeded to get acceptance.
- JGI will be looking for new sequencing projects soon.
- JGI and other government research labs are under severe financial stress right now.
- DOEs mission is targeted at carbon sequestration, phyto-remediation, and biofuels. We should be able to hit at least one of these!

In short, there appears to be a reasonable window of opportunity and we should be able to make a pretty compelling argument that loblolly pine is a good candidate for sequencing. Additional BAC library resources may be needed.

Five Year Research Plan: Objectives and Action Steps

Within the next 5 years, this sub-committee recommends pursuit of the following objectives and their associated action steps.

Objective	Action Steps	Party Responsible	By Date
Create 3 cloned full-sib families (3-generation) for replicated site deployment to serve as community QTL mapping populations.	• Approach 3 pine TI Cooperatives to establish 1 population each. Provide technical assistance for planning.		9/1/03
Place 350 SSR markers on pine reference maps.	• Develop 250 new markers, optimize and map a total of 350	USFS Southern St.	6/1/04
Place framework SSR and comparative mapping markers on new community QTL populations.	• Same as objective		2006
Encourage phenotypic assessments of all types on these populations.	• Host a website and a conference to encourage non-molecular partners (physiologists, etc) to collaborate. • Seek support from Agenda 2020		2005
Place 3K to 5k members of a uni-gene set on reference map	• Develop funding strategy for large-scale gene mapping project.		6/04
Sequence a portion of the pine genome	• Develop a position paper seeking support at JGI for sequencing 0.05 of the pine genome.		10/03

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Table 1. Peer-reviewed forest tree QTL citations, study parameters, and species/traits evaluated.

***Taxa* Studied** **Trait**	**Objective**	**Population**[1]	**Number of Progeny**	**Number of Markers**	**Marker Type**[2]	**# of QTL/trait** Range variance explained/qtl **Total variance explained/trait**[3]	**Citation**
Pinus taeda **Specific gravity;**	**Detection**	**F_2 OB**	**177**	**75 – 87**	**RFLP**	**5 QTL** **23% P**	**Groover et al. 1994**
Pinus pinaster **Growth, growth components**	**Detection;** **Stability;** **(age)**	**F_2 S**	**126**	**120**	**RAPD**	**1 – 3 QTL** **5 – 14 %** **6 – 24 % P**	**Plomion et al. 1996a**
Pinus pinaster **Monoterpene**	**Detection**	**F_2 S**	**126**	**120**	**RAPD**	**1 QTL** **26.5 % P**	**Plomion et al. 1996b**
Pinus radiata **Cone production, branching, diameter**	**Detection**	**F_1OB**	**134**	**?**	**RAPD**	**?**	**Aitken et al. 1997**
Pinus taeda **Wood density**	**Detection Methods**	**F_2 OB**	**172**	**75 –87**	**RFLP**	**1 QTL** **8% P**	**Knott et al. 1997**
Pinus palustris*elliottii **Early ht growth**	**Detection**	**F_1 OB**	**120**	**?**	**RAPD**	**?**	**Kubisiak et al. 1997a**
Pinus radiata **Growth**	**Detection** **Stability(age)**	**F_1 OB**		**222**	**RAPD**	**2 QTL** **9 – 10%**	**Emebiri et al. 1998**

[1] F_1OB = two generation outbred; F_2OB = three-generation outbred; F_1IB = three-generation inbred; F2S = three generation self-fertilization; F1S= two-generation self-fertilization; HS= half-sib;

[2] RFLP = restriction fragment length polymorphism; RAPD=random amplified polymorphic DNA; STS=sequenced tag site; AFLP=amplified fragment length polymorphism; SSR=simple sequence repeat

[3] P=phenotypic variance explained; G=genetic variance explained

Pinus elliottii **Aluminum tolerance**	**Detection**	**F_1 OB**	**186**	**?**	**RAPD**	**?**	**Kubisiak et al. 1999**
Pinus radiata **Wood density**	**Detection Stability (ring location)**	**F_1 OB**	**93**	**126**	**RAPD, AFLP, SSR**	**1 –2 QTL**	**Kumar et al. 2000**
Cryptomeria japonica **MOE**	**Detection**	**F_1 OB**	**72**	**84 – 119**	**RAPD**	**15 QTL** **45% P**	**Kuramoto et al. 2000**
Pinus sylvestris **Bud set; frost hardiness**	**Detection Methods Stability**	**F_1 full sib (Backcross)**	**84**	**164**	**RAPD**	**1 – 8 QTL** **3-13%** **3 – 25% P**	**Hurme et al. 2000**
Pinus sylvestris **Frost hardiness;wood density; branch diameter; growth;**	**Detection**	**F_1 OB**	**94**	**94 – 155**	**AFLP**	**0 – 3 QTL** **9 – 23% P**	**Lerceteau et al. 2000**
P*inus radiata* **Inbreeding depression; Survival;**	**Detection Architecture**	**F_1S**	**378 seed**	**54 – 202**	**RAPD, SSR**	**9 regions** **sub-lethal to lethal**	**Kuang et al. 1999**
Pinus taeda **Inbreeding depression; Survival and growth**	**Detection Architecture**	**F_1S**	**373 seed**	**Hundreds**	**AFLP**	**3 – 4 QTL** **2 – 14% P**	**Remington and O'Malley. 2000**
Pinus taeda **Growth;**	**Detection Stability (Age, genotype)**	**F_2OB**	**84 – 171**	**62 – 173**	**RFLP, RAPD**	**1 –3 QTL** **7 – 59% P**	**Kaya et al. 1999**

Pinus taeda **Wood density; Microfibril angle; % late wood;**	**Detection Stability (Age)**	**F_2OB**	**172**	**109 - 164**	**RFLP,**	**5 – 9 QTL** **5- 15% P**	**Sewell et al. 2000**
Pinus taeda **Wood chemistry traits;**	**Detection Stability (Site)**	**F_2OB**	**165**	**109 - 164**	**RFLP,**	**8 QTL** **5 – 13% P**	**Sewell et al. 2001**
Pinus taeda Wood physical and chemical properties;	**Verification (cohort, site, family stability)**	**F_2OB**	**450**	**109 – 164**	**RFLP, ESTp**	**5 – 12 QTL** 1.7 to 15.9% P	**Brown et al. 2003**
Pseudotsuga menziesii **Vegetative phenology;**	**Detection Stability (Site, age)**	**F_2OB**	**78 – 224 clonal replicate**	**74**	**RFLP,**	**2 –4 QTL** **2 – 11%** **8– 36% P**	**Jermstad et al. 2001 a**
Psuedotsuga mensziesii **Cold hardiness;**	**Detection**	**F_2OB**	**190 clonal replicate**	**74**	**RFLP,**	**2 - 6 QTL** **2 – 10%** **~7 – 25% P**	**Jermstad et al. 2001 b**
Pseudotsuga menziesii **Vegetative phenology;**	**Verification (cohort, site, family stability)**	**F_2OB**	**470 clonal replicate**	**74**	**RFLP,**	**4-11 QTL** 2 – 9.5%	**Jermstad et al. 2003**

Genetic Stocks And Germplasm Subcommittee Report

Submitted by: Barry Goldfarb and Tim Mullin
Team: Brian Baltunis (UF), Barry Goldfarb (NCSU), Joe Nairn (UGA), Tim Mullin (NCSU)

1. Resources available

1.1. Genetic stocks

1.1.1. Several laboratories have compiled collections of DNA samples. Existing DNA collections fall into two categories—DNA of cloned genes or genomic DNA of populations of trees. In most cases, collections are maintained by individual laboratories, however, in some cases, collections have been duplicated and stored in more than one laboratory.

1.1.1.1. Examples of DNA collections of cloned genes follow:

1.1.1.1.1. NCSU/Sederoff et al--NSF – ESTs, largely from xylem

1.1.1.1.2. ADEPT/Davis, Covert et al – ~400 genes differentially expressed during disease interactions, with plans to expand to ~5,000 in the near future.

1.1.1.1.3. UGA/Dean et al-- NSF – ESTs, largely from roots of plants exposed to different levels of drought stress, other tissues/conditions to be added as become possible

1.1.1.1.4. IPST/Cairney et al--NSF – ESTs, from embryogenesis, both zygotic and somatic

1.1.1.2. Examples of DNA collections from populations of trees follow:

1.1.1.2.1. UC-Davis/Neale – reference mapping population: 96 3rd-gen trees from WeyCo, parents and grandparents

1.1.1.2.2. ADEPT/UC-Davis/Neale et al—DNA from 1400 clones (from FBRC CCLONES study) from ~60 full-sib crosses

1.1.1.2.3. Neale/Goldfarb/Loopstra—Megagametophytes and diploid tissue (DNA not yet extracted) from 500 unrelated individuals from across the loblolly pine range (from NCSU and Western Gulf Tree Improvement Programs)

1.2. Germplasm. Loblolly pine is one of the few species for which germplasm is available both in widely distributed natural populations and in economically based breeding programs. This is largely the result of the relatively undomesticated status of the species, but it represents a unique scientific opportunity.

1.2.1. Natural populations

1.2.1.1. Natural populations of loblolly pine still exist, although most of the region underwent significant disturbance as a result of agricultural land clearing. The natural populations that exist now are largely the result of seeding in on abandoned farms. As time passes, these natural populations are getting smaller and fewer. In some cases, the availability of natural populations in certain regions may be threatened. Because of the large size of the natural population, it is unlikely that many important alleles have so far been lost

1.2.2. Breeding populations

1.2.2.1. Commercial breeding populations are maintained by the members—both private companies and state agencies—of the three major breeding cooperatives, NCSU-ICTIP, WGTIP (Texas FS), and CFGRP (UF). These populations have been primarily selected for rapid growth, straight boles, and to some extent, fusiform rust resistance. They do not constitute a random sample of pre-selection populations.

1.2.2.2. The trees with higher breeding values (commercial worth) are generally well archived for genetic conservation. However, less commercially valuable trees are less well archived and some have already been lost. Formal genetic conservation strategies vary among the coops and their members, but more effort is expended on commercially valuable material than on material without known commercial value.

1.2.2.3. It is highly probable that most, if not all, of the original gene diversity is still represented in these selected populations. Common alleles should be well conserved, while the fate of rare alleles (< 1%) is less certain.

1.2.3. Seed storage

1.2.3.1. There is an indication that some loblolly pine seed is stored at the National Seed Center, however, the extent and nature of the curated collection is not known. Some seed is stored at the facilities of some of the breeding coops, although these tend to be small collections during active breeding and testing or specialized research collections. Most of the seed is stored by individual organizations and, again, there is a heavy emphasis on commercially valuable seed by today's standards.

1.2.4. Ability to conserve and produce germplasm

1.2.4.1. Seedlings: many organizations, including private companies and state agencies operate commercial nurseries that produce bare-root loblolly pine seedlings (>1 billion produced annually). In addition, on a smaller scale, there are commercial containerized seedling producers and numerous companies, agencies and universities produce seedlings (usually containerized) for research studies. Thus, given high-quality seed, there are numerous options for producing quality seedling stock.

1.2.4.2. Cloning: One very useful tool for studying genomics of loblolly pine is the ability to clone individual genotypes. By replicated trees of a given genotype a much more precise estimate may be made of the genetic vs. environmental contributions to gene expression and phenotype. Currently, there are two relatively common methods for cloning loblolly pine trees--rooting stem cuttings and somatic embryogenesis. Both methods rely on starting from juvenile material, thus, mature genotypes may not be cloned. In addition, it is possible (and relatively routine) to multiply the shoot system (but not the roots) of mature trees by grafting (not considered further here)

1.2.4.2.1. Rooted cuttings: The technology to root stem cuttings is not exceptionally difficult to achieve, although it does require some

specialized facilities and some experience. Currently, this technology is well established at NCSU and in several companies. The ability to propagate loblolly pine by rooted cuttings extends to most genotypes. However, in a short period of time, relatively few copies (ramets) of a given genotype can be produced. In addition, after clones are maintained for a number of years, maturation may affect gene expression and phenotypic expression.

1.2.4.2.2. Somatic embryogenesis: This technology is more specialized and requires tissue culture facilities and extensive know-how. Currently, while there is research underway in the public domain to improve the process, there are no public facilities or labs that can routinely produce clones by this method. There are however, at least two private companies with extensive embryogenesis facilities and expertise. In general, the limitation to embryogenesis from a genomics perspective is that a fair proportion of genotypes seem to be recalcitrant to propagation by this method. On the other hand, fairly large numbers of ramets of a given clone can be produced once a clone has successfully initiated an embryogenic culture. Moreover, cultures can be stored essentially indefinitely in liquid nitrogen. This can be useful for germplasm storage purposes and for controlling maturation in clones to be used for genomic studies over long periods of time.

1.2.5. Scientific study populations

1.2.5.1. CCLONES (FBRC/ADEPT)--~1400 clones from approximately 60 full-sib crosses, originally from the Lower Gulf Elite Breeding Population—a joint effort of the three breeding coops. X number of ramets were planted in XXXX on X sites.

1.2.5.2. Neale/Weyco reference mapping population—Pedigree? Details? Location?

1.2.5.3. Neale/Loopstra/Goldfarb association population—500 individuals from across the entire range of loblolly pine—set up to be an association mapping population. Seed obtained from the NCSU and Western Gulf coops. Currently, these exist as seedlings at NCSU, but they will soon be pruned into hedges (stock plants for rooted cutting production) and a limited number of ramets produced.

2. What's needed

2.1. <u>Genetic stocks</u>

2.1.1. Ideally, there would be an infrastructure for a centralized, curated collection. Critically important, would be the distribution mechanism, which is an ongoing and resource-consuming task. It might not be ideal for this to be done privately, as the costs would need to be high to provide a profit margin and there would be no guarantee about stability of a private firm. Therefore, a publicly funded entity would be best. Because of the service function, this might not be appealing to a research laboratory, unless there was sufficient funding for it to be self-sustaining.

2.2. Germplasm

2.2.1. Ideally, a common set, or several common sets of plant material would be publicly available for research purposes. This would facilitate cross-referencing and integration across different studies and among different investigators. Germplasm can be maintained as seeds, as grafts (shoot system only), as hedged stock plants for rooted cuttings, or as cryopreserved somatic embryogenic cultures. The latter two options allow the possibility of the genotypes being propagated for new field or other phenotypic studies.

2.3. Gene conservation for Southern pines

2.3.1. To preserve genotypes containing rare, or non-commercial alleles, an ex situ seed storage program would be an excellent asset. Terms for public or scientific community access would have to be arranged in advance. The US National Seed Center might serve a seed storage function.

2.3.2. For commercial populations, an integrated strategy involving the three breeding cooperatives and their members would be ideal.

2.4. Propagation/Transformation

2.4.1. Rooted cutting propagation. While several private companies have the capability for producing large number of rooted cuttings, and universities could develop such a capability, currently only NCSU has the facilities and expertise to accomplish this type of plant production for research studies. Because of the time required to produce cloned trees of specific genotypes, advance planning and infrastructure support would be an asset.

2.4.2. Somatic embryogenesis. Routine use of this technology by the research community would be advantageous for two principal reasons: (1) it would allow for indefinite storage of genotypes (cryopreservation) in a form capable of producing new plants (embryogenic cultures) and (2) it is a platform for genetic transformation (see next section). Many advances in embryogenesis know-how have been made by the private sector. Greater collaboration between commercial entities and the public research community would be beneficial.

2.4.3. Genetic transformation. The lack of a routine, publicly available, genetic transformation system for loblolly pine remains a serious impediment to functional genomics research progress, as well as the ability of individual researchers to attract grant funding. However, grant funds from federal, competitive agencies to develop transformation technology are not likely. As with somatic embryogenesis, substantial advances have recently been made in the private sector. Closer cooperation among public researchers studying or using transformation and greater collaboration with private firms with transformation expertise would benefit the field.

2.5. Information access. Research on loblolly pine genomics would be facilitated by increased and more efficient access to information. Comprehensive relational databases would allow researchers working on the same genotypes to exchange information and make the availability of plant material more accessible to potential researchers. Because there are various ownerships of the germplasm, relational databases would need to protect confidential information, while making public information freely available. Public researchers would benefit

from having more availability to relevant plant material and private owners of the germplasm would benefit from increased information about their plant material.

3. Essentials of Five-Year Plan

3.1. Genetic stocks. A curator should be nominated to maintain and distribute DNA stocks. Funding from a public agency should be sought.

3.2. Germplasm conservation

3.2.1. Work with USDA Seed Lab and/or Forest Service to develop and implement comprehensive ex situ conservation by seed

3.2.2. Work with breeding coops and their members to develop comprehensive strategy for archiving, lists of desirable genotypes (including public accessible genotypes), and specific plan for establishing or expanding clone banks

3.2.2.1. 96-clone WeyCo population should be reproduced at 2nd (3rd) site

3.3. Plant Production/Propagation

3.3.1. Rooted cuttings. Plan ahead for cloning of populations for genomic studies.

3.3.2. Somatic embryogenesis: Promote collaborations with private companies for culture intitiation, cryopreservation, plant production

3.3.3. Transformation.

3.3.3.1. Establish a public domain "SE Transformation Network" to encourage collaborative efforts among academic researchers

3.3.3.2. Seek collaborations with private companies for high-throughput transformation for functional genomics studies.

3.4. Information Access

3.4.1. Begin to migrate and integrate Tree Improvement Coop databases with Bioinformatics relational database with appropriate access controls

3.4.1.1. Build a metadatabase with sophisticated data collection and sharing tools

3.4.2. Facilitate the use of Material Transfer Agreements by drafting a general format appropriate for pine materials that clarifies appropriate conditions for publication of genotype identification, commercialization of genotypes, genes, or gene products, and other intellectual property issues among public researchers and private owners of genetic material.

Appendix 1 – List of Participants

Texas A&M University
- Dr. Tom Byram*
- *Dr. Carol Loopstra**

University of Florida
- Dr. John Davis*
- Dr. Alison Morse
- Gogce Kayihan
- Brian Baltunis
- Dr. Gary Peter*
- *Dr. Tim White**

University of Georgia
- Dr. Jeff Dean*
- Dr. Marie-Michele Cordonnier*
- Pratt
- Dr. Lee Pratt*
- Dr. Joe Nairn*
- Kimberly Hunt
- *Dr. Sarah Covert**

University of California, Davis and USFS Pacific SW Station
- Dr. David Neale*
- Dr. Garth Brown*
- Dr. Richard Michelmore
- Dr. Nicholas Wheeler* (Consultant)

North Carolina State University
- Dr. Tim Mullin*
- Matias Kirst
- Dr. Barry Goldfarb*
- *Dr. Len van Zyl**
- *Dr. Ron Sederoff**

Institute of Paper Science Technology
- Dr. John Cairney*
- *Dr. Ulrika Egertsdotter**

University of Minnesota
- *Dr. Ernie Retzel**

Weyerhaeuser Company
- Edgar Fuchs

USFS Southern Research Station
- Dr. Craig Echt*
- *Dr. Dana Nelson**

Names in italics invited but unable to attend
*** Mailing list**

Appendix 2: Gary Peter notes

A. Unique features of the Pines