What do we know about the genetic determinism of cavitation resistance in maritime pine?

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Session 1: Traits for adaptation/Looking for the adaptive response

Invited Talk

Disentangling the roles of history and local selection in shaping clinal variation of allele frequencies and gene expression in spruce species.

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Understanding the genetic basis of local adaptation is challenging due to the subtle balance among conflicting evolutionary forces that are involved in its establishment and maintenance. One system with which to tease apart these difficulties are clines in adaptive characters. Here we analyzed genetic and phenotypic variation in bud set, a highly heritable and adaptive trait, along two clines, one in Norway spruce (*Picea abies*) and the other in Siberian spruce (*P. obovata*). In Norway spruce clinal variation was analyzed among 18 populations, arrayed along a latitudinal gradient ranging from 47°N to 68°N. We confirmed that variation in bud set is strongly clinal using a subset of five populations. Genotypes for 137 single nucleotide polymorphisms (SNPs) chosen from 18 candidate genes putatively affecting bud set, and 308 control SNPs chosen from 264 random genes, were analyzed for patterns of genetic structure and correlation to environment. Population genetic structure was low (F_{ST} = 0.05), but latitudinal patterns were apparent among Scandinavian populations. Hence, part of the observed clinal variation should be attributable to population demography. Conditional on patterns of genetic structure, there was enrichment of SNPs within candidate genes for correlations with latitude. Twenty-nine SNPs were also outliers with respect to F_{ST}. The enrichment for clinal variation at SNPs within candidate genes (i.e. SNPs in Pa*GI*, Pa*PhyP*, Pa*PhyN*, Pa*PRR7* and Pa*FTL2*) indicated that local selection in the 18 populations, and/or selection in the ancestral populations from which they were recently derived, shaped the observed cline. Validation of these genes using expression studies also revealed that Pa*FTL2* expression is significantly associated with latitude, thereby confirming the central role played by this gene in the control of phenology in plants. Additionally, we also validated these results with six populations of Siberian spruce (*Picea obovata*), arrayed along a latitudinal gradient ranging from 55°N to 67°N. Nine of the top candidate genes in the Norway spruce study were resequenced in around 100 individuals, for a total of 29,000 bp. Population structure was assessed with 14 SSR loci and 80 silent sites. We detected significant clinal variation and/or evidence of local selection at some of the same candidate genes already picked in Norway spruce. This is unlikely to be due to population history as there is an almost complete absence of population structure.

The research leading to these results has received funding from the European Community's Seventh Framework Programme (FP7/ 2007-2013) under the grant agreement n° 211868 (Project Noveltree)

Oral presentations

Population genetic structure, plant material certification and climate adaptation in maritime pine breeding zones.

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Population genetic structure and molecular footprints of adaptation to diverse climatic conditions were investigated in native maritime pine populations encompassing distinct breeding zones. The present study involved the scanning of 12 nuclear microsatelites (nuSSRs) and 384 single nucleotide polymorphisms (SNPs) distributed in 221 genes, the later including a number of amplicons potentially targeted by selective forces, as reported in previous functional, association and population genetic studies in conifers. Overall patterns of neutral population structure revealed six clear genetic clusters, identified through principal component and Bayesian clustering analyses on nuclear microsatellites and SNPs. These groups mostly coincided with those previously reported using cytoplasmic DNA markers (but were not identical) and were distributed in Atlantic France, Corsica, Morocco and the Atlantic (plus Tunisia), Central and Mediterranean regions of the Iberian Peninsula, respectively. A high-throughput certification test (based on SNPs) to identify reproductive materials from the Iberian and French Atlantic regions, where major breeding programs are in place, was developed and used to identify plantations from other origins in these regions. Neutral molecular data was also used to remove confounding effects of shared ancestry (i.e. populations derived from the same gene pool) in genotype-environment correlations (as in Eckert *et al*. 2010; Grivet *et al*. 2011). Significant genotype-environment associations were identified in about 2% of the surveyed gene SNPs. A more in-depth scan of the Spanish populations located in the Atlantic and Mediterranean breeding regions revealed a few more polymorphisms putatively responding to contrasting selective pressures. Most significant associations were found in the Mediterranean range of the species, which has also a more heterogeneous environment. Remarkably, most of these associations involved temperature indices during both the winter and the summer seasons, but not rainfall. The functional annotations of the genes carrying these environmental responsive SNPs supported their implication in adaptation to extreme temperatures. Alleles and candidate genes involved in maritime pine adaptive responses to climate will be useful to develop molecular breeding applications in this species.

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Genome-wide SNP detection and genetic population inferences on 52 *P. nigra* genotypes resequenced by next-generation sequencing.

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Background

The challenges facing forest geneticists and tree breeders include recognizing high value forest products and promoting sustainability of forest ecosystems under climate change. A suitable approach to achieve these goals is the use of genomic tools to understand and exploit the genetic variability requested to generate the different responses to the environment.
In the framework of a joint resequencing effort undertaken by the EU projects Evoltree, Noveltree, and EnergyPoplar, genome-wide genetic variability has been investigated in *Populus nigra*, the native and most wide-spread poplar species in Europe. In addition to different genetic resources available for the genus *Populus*, *i.e.* mapping pedigrees, association populations, genetic and QTL maps as well as the *P. trichocarpa* genome sequence, the present work aims to provide a large amount of single nucleotide polymorphisms (SNPs) spread across the whole black poplar genome, to be used as valuable genomic resource in novel breeding programs. The exploitation of next-generation sequencing technologies allowed us to easily reach the objective.

Methods

Using the Illumina technology we sequenced fifty-two natural genotypes selected to represent the European latitude range. Four of these, belonging to different latitudinal settings, were resequenced at a high coverage (about 20X each) in order to obtain a dataset of informative SNPs. SNP detection was based on a reference-guided assembly using the *P. trichocarpa* genome sequence. Test experiments showed the feasibility of using the latter genome sequence as reference for *P. nigra*, given that 75% of *P. nigra* reads were uniquely mapped on the *P. trichocarpa* sequence. Data validation was performed using as gold standard a dataset of more than 96000 loci generated by Sanger amplicon resequencing, which literature refers to provide a 7% of False Discovery Rate (FDR) compared to microarray genotype calls. Different aligners (BWA, CLC) and base callers (VarScan, CLC) were tested to select the best performing combination of aligner/base-caller. Read quality filtering and stringency on the detection parameters proved effective in reducing the FDR of our SNP detection to values around 5%, and allowed us to use the informative SNP dataset for genetic population inferences. In a second instance, given the bioinformatics strategy achieved in the previous whole-genome study on few high-covered genotypes, the SNP detection was extended to sequence reads obtained from 48 additional European clones, which were resequenced at low coverage (spanning 2 to 10X) in order to maximize the detectable variability of the poplar European populations. Due to the low coverage, the resequencing was performed using indexed Illumina libraries to check reads belonging to each individual, but alignments and SNP detection were performed on the whole set of reads obtained.

Results and Discussion

Based on the four high-coverage clone data, estimates of polymorphism frequency and nucleotide diversity showed that *Populus nigra* is more variable (nucleotide diversity of 0.0071 across its whole genome) than most forest trees and crop species and only less variable than European aspen and sugar beet. A clear correlation was observed among the distribution of the nucleotide diversity, the genes and the repetitive regions across the genome: regions with higher gene content and lower repetitive sequences present lower nucleotide diversity estimates. Then, we used the dataset of SNPs detected in the 48-clone panel to confirm the informative SNPs and evaluate their power to describe the genetic variation in *P. nigra*.

We are now working on a procedure to extract reliable SNP allele frequencies from the different datasets, in order to use them in genetic population analyses at genome level. In particular, we will perform clusterization and differentiation analyses of the whole set of 52 individuals and compare them to the corresponding analyses performed using microsatellites, considered as control. The potential and the drawbacks of using high-coverage small panels or low-coverage large panels in such analyses will be addressed.

Conclusions

A lot of resequencing studies have been performed in the past in different forest tree species to unravel the genetic variation responsible for phenotype shaping. All that works focused their analysis effort to a set of candidate genes or a few candidate genomic regions resequenced in a panel of 12-to-100 genotypes. In this work we focused on the potential of whole-genome resequencing data to get information in terms of genetic variability and genetic population aspects at a genome level. This is still a challenging issue due to the amount of sequence data requested together with the computational resources and bioinformatics skills needed to compute such data. With this work we provided an approach and a validated procedure to detect polymorphisms at a genome-wide scale. We also provided a procedure to use these polymorphisms to infer site frequency, nucleotide diversity and differentiation estimates.

Key words:

Genetic variability, population differentiation, next-generation sequencing, Populus.

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Evolution of diversity in Prunus avium: assembly of its genome using Next Generation Sequencing.

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Sweet cherry (*Prunus avium*) is an economically important crop that belongs to the genus *Prunus* along with almonds, peaches, plums, and apricots. Its diploid genome is organized in 8 chromosomes and has an estimated size of 338 Mb. Recently, the whole genome sequence of the related species *Prunus persica* has been released marking a turning point for the genomic studies of this genus. In the framework of a national project aimed at the study of the effects of domestication on genetic variability in cherry, we set out to build a first draft genome sequence of *Prunus avium* by means of next-generation sequencing. We sequenced several paired-end libraries of the sweet cherry variety *Big Star* using the Illumina HiSeq 2000 platform at a coverage of approximately 100X. CLC Genomics Workbench was employed for the *de novo* assembly of the short reads in 43,011 contigs having an average size of 4.7 Kb, an N50 size of about 14 Kb and covering in total 204 Mb (about 60% of the estimated genome size). Mate-paired libraries of 2.5 Kb where prepared following a hybrid 454-Illumina protocol and were used to perform the scaffolding. A preliminary assembly composed by 30,204 scaffolds with an average size of 6.8 Kb and an N50 size of about 29 Kb was obtained. A first comparative analysis between the *P. avium* and the *P. persica* genomes was performed by aligning the *P. avium* scaffolds to the *P. persica* reference genome. Overall, about 105 Mb (~51% of the assembly) were aligned to the *P. persica* genome with an identity higher than 80% suggesting a high level of conservation between the two species. Conserved sequences showed in general a high gene content and a low rate of repetitiveness. The *P. avium* genome assembly will be further refined and improved and will provide a new resource for biological research and breeding of this species. The future aim of the project will be the resequencing of several local landraces, commercial varieties and wild *P. avium* genotypes to explore the genetic mechanisms influencing domestication in this species at a genome-wide level.

Adaptive evolution in the Eurasian Scots pine with comparison to the Mediterranean maritime pine: two conifers with contrasted distribution and demographic history.

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Comparing adaptive strategies of species with common and contrasted characteristics in terms of distribution, demographic, and life histories is useful to detect common or individualistic evolutionary patterns, and tease apart the evolutionary forces at stake. Here we analyze the pattern of sequence variation in Scots pine (*Pinus sylvestris*, Subsection Pinus) and maritime pine (*Pinus pinaster*, Subsection Pinaster), two conifers with large population effective size and characterized by ancient bottleneck histories, but that live in distinct areas and were distinctly affected by glaciations. Cold-tolerant *P. sylvestris* is widely distributed in Europe and central Asia, while more xeric *P. pinaster* is restricted to the western Mediterranean basin. The species also have contrasted population genetic differentiation (low for *P. sylvestris*, high for *P. pinaster*).
We used a common set of 355 genes or gene fragments, including twenty phenology and dehydration-response genes, analyzed across the range distribution of the two species, and performed several tests based on synonymous and nonsynonymous substitutions to detect selection events. Our specific objectives were: i) to compare the pattern of genetic variation between the two species using the same amplicons, ii) to detect genes that are under selection, either specific to one species or common to the two species, iii) to estimate the proportion of nonsynonymous divergence driven by positive selection (alpha) and the distribution of fitness effects (DFE) of new mutations, by jointly inferring selective and demographic effects, and iv) to assess the effects of different levels of population structure (stronger in maritime pine) on alpha and DFE.
Results from the DFE adaptive test showed that, for the two species, the demographic model that fitted better the distribution of neutral substitutions (i.e. synonymous substitutions) and adaptive substitutions (i.e. nonsynonymous substitutions) was population size change (i.e. a bottleneck) with adaptation. Moreover, *P. sylvestris* showed lower proportion of adaptive substitutions than *P. pinaster*, and though for both species the majority of nonsynonymous mutations were strongly deleterious, this trend was stronger for *P. pinaster* than for *P. sylvestris*. By contrast, the McDonald-Kreitman Poisson Random Field (MKPRF) test showed a higher proportion of genes under selection in *P. sylvestris* than in *P. pinaster*. Actually, from the twenty phenology and dehydration-stress candidate genes, three (*CoL1*, *Dhn2* and *Dhn5*) showed signs of positive selection and two (*Dhn1* and the putative *glutathione S-transferase* coding locus 0_4042_01) showed signs of negative selection in *P. sylvestris*, while in *P. pinaster*, only negative selection on *Dhn1* was observed. We discuss the different patterns detected by the tests performed.
These results give new insights into understanding the origin of molecular variation at candidate genes in the two pine species, as well as their mechanisms for adaptation to their respective environments.

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Adaptive variation and genetic architecture of male and female reproductive traits in *Pinus pinaster*.

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The mechanisms of species adaptation to their environment can be seen as a complex net of interrelations between processes, where conflicting compromises exist. Reproduction, due to its direct link to fitness plays a major role in explaining adaptive strategies. Moreover, reproduction can be accomplished by either female or male functions and the balance between costs and benefits of female and male reproduction can show different patterns along development and heterogeneous and changing environments.
Pinus pinaster (Maritime pine) is an economically important tree species in Southwestern Europe, than can be found in varying ecological conditions (substrate, elevation, climate). Its broad ecological niche and adaptive genetic diversity makes it an interesting study case for the differential adaptive role of early male and female reproduction. A deeper knowledge of adaptive mechanisms related to reproduction will also be useful to improve the resilience of afforested lands.
However, reproductive data are typically coded as binary (present/absent) or discrete (counts) variables, which hampers a straightforward use of conventional statistic tools for quantitative genetics. Increasingly popular Generalized Linear Mixed Models (GLMMs) are suitable for the analysis of non-Gaussian data, although quantitative genetic parameters provided by these models are very rarely reported.
Here, we deepen into the genetic architecture of threshold traits and ask whether *P. pinaster* shows differences in the threshold size at first reproduction through male versus female function among populations and if those differences are related to the environment of origin.
In our work, we used data from range-wide *Pinus pinaster* populations and progenies grown in common garden experiments. Generalized Linear Mixed Models fitted by MCMC were applied to Gaussian and non-Gaussian data. This allowed us to test the existence of genetic differentiation and compute quantitative genetic parameters for traits such as the threshold size for reproduction and the number of reproductive structures in either sexual function. Standard Linear Mixed Models were used for other traits with Gaussian distributions like height.
Significant variation at the population level was found for the threshold size for reproduction either via male or female functions, as well as the sex allocation (male/female investment) at the onset of reproduction. Female reproductive function showed a delayed ontogeny and higher variability (229.5-490.4 cm threshold size range) compared to male function (208.6-283.6 cm threshold size range). A lower variability of male reproduction was also reflected in a lower heritability; however, both heritabilities for male and female traits were higher than heritability for growth. Provenances from milder Atlantic climate showed a more vigorous growth and male-biased reproduction, while populations that evolved in harsh Mediterranean climates attained lower sizes and higher investment in female reproduction.
Our results in *P. pinaster* highlight a major role of female reproduction in local adaptation patterns, as revealed by the ecotypic trend of variation found, even after accounting for neutral genetic structure. The relevance of female reproduction is also shown by negative genetic correlations with growth, a trait that is widely acknowledged as adaptive. At the same time, despite likely strong natural selection on reproductive traits, we found high values of among-family variation. This is indicative of a high potential to evolve naturally or to be modified by artificial selection, which is relevant under a new paradigm of forest breeding aimed at resilience.

Plasticity versus stability (or lack of it): when scientific fields collide.

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Phenotypic plasticity has been a trending topic in biological research for the last decades. In plant science in particular, the uncertainties due to climate change have driven increasing attention on this concept, searching for a more complete view of the adaptive potential of plant populations facing too rapid changes for genetic change or migration. Recent scientific literature has dealt with virtually all aspects and levels of plasticity in plants: from developmental to physiological, and from species or populations down to gene expression. However, we still know too little about how to use this variety of plastic responses, and still a question arises about whether plant breeders should search plastic or less plastic phenotypes. We know that plasticity is both trait- and genotype- specific, and also that the precise environmental range might enhance or hinder the expression of plasticity for the same trait x genotype combination. Moreover, lack of plasticity in one level under changing environment implies underlying plastic physiological mechanisms and vice versa.
In particular, one of the major problems detected after a specific methodological workshop and compiling different experiments in NovelTree project, is the mismatch between several methods focused in dissecting GxE interaction (Ecovalence, AMMI, etc.) and those focused on specific plasticity values per genotype (plasticity indexes, JRA, etc). The first ones -widely used in breeding- are highly suitable for identifying the *stable* genotypes, meaning those reacting to a given environmental range in a predictable way, i.e., close to the average of all studied genotypes. Therefore, a *stable* genotype under this point of view is not equivalent to a *non-plastic* genotype, but rather to a genotype that is *as plastic as the average*. In this communication we review data from different experiments and compare stability vs. plasticity after different methods intending a disambiguation of both concepts as a necessary step for going ahead in the comprehension of this too flexible concept.

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Posters

Adaptive evolution of Mediterranean pines.

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Mediterranean pines represent an extremely heterogeneous and interesting assembly. Although they have evolved under similar environmental conditions, they diversified long ago, *circa* 10 Ma, and present distinct biogeographic and demographic histories. Therefore, it is of special interest to understand whether and to what extent they have developed specific strategies of adaptive evolution through time and space. To explore evolutionary patterns, the Mediterranean pines' phylogeny was first reconstructed analyzing a new set of 21 low-copy nuclear genes with multilocus Bayesian tree reconstruction methods. Secondly, a phylogenetic approach was used to search for footprints of natural selection and to examine the evolution of multiple phenotypic traits. We identified two genes (involved in pines' defense and stress responses) that have likely played a role in the adaptation of Mediterranean pines to their environment. At the phenotypic levels, few life-history traits show historical or evolutionary adaptive convergence in Mediterranean lineages. Finally, pattern of character evolution revealed various evolutionary trade-offs linking growth-development, reproduction and fire-related traits. Assessing the evolutionary path of important life-history traits, as well as the genomic basis of adaptive variation is central to understand the past evolutionary success of Mediterranean pines and their future response to environmental changes.

Adaptation of *Taxus baccata* to environmental gradients: selection patterns in Taxaceae transcriptomes as a tool to select candidate adaptive genes.

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European yew (*Taxus baccata* L.) is a long-lived coniferous species, whose populations cover a very wide geographic range (from European Atlantic coasts to Scandinavia and the Middle-East) and a great variety of environmental and climatic conditions. As other *Taxus* species, European yew is acknowledged for its production of antitumoral diterpenes (e.g. taxol), and is now protected in most European countries. In spite of high dispersal potential, English yew shows strong genetic structure, both within and among populations (Dubreuil *et al.* 2010, Gonzaléz-Martínez *et al*. 2010, Chybicki *et al*. 2011). The progressive warming of climate since the Last Glacial Maximum and the impact of human activities (e.g. logging, fires) seem also to have contributed to a persistent fragmentation and effective size reduction of *T. baccata* populations, decreasing gene flow, and increasing genetic drift and inbreeding.
Our knowledge of the demographical history of *T. baccata* in Europe raises the question of its ability to adapt to new environmental changes, especially related to climate (e.g. increase of drought and extreme temperatures, and bigger and more frequent forest fires). Preliminary results from common garden experiments reveal high phenotypic variability, as well as overall patterns of growth and functional traits associated with climatic clines (temperature and precipitation), suggesting local or regional adaptation. Selective footprints have also been detected in some taxol-related genes for Iberian *T. baccata* populations (Burgarella *et al.*, 2012).
To further document the adaptation processes that European yew populations may have undergone at the molecular level, we are currently developing a high-throughput genome-wide sequencing approach. To dissect molecular patterns of adaptation to environmental clines, a first step is to select suitable candidate genes for adaptive traits. With this purpose, we obtained the nearly-complete transcriptome of *T. baccata* using Illumina sequencing technology. After assembly and trimming, we produced a functional annotation focusing on coniferous orthologs (using FullLengtherNext, at SCBI www.scbi.uma.es/ingebiol/session/new/full_lengther_next) for ca. 17,000 *T. baccata* unigenes. These were further aligned and compared to other Taxaceae transcriptome unigene datasets (*T. mairei*, *T. cuspidata*) as well as to a set of full-length cDNAs in *Picea sitchensis*, to calculate ratios of non-synonymous to synonymous substitution rates across species (ω=dN/dS) and thus detect selection signatures at particular candidate gene loci.
Altogether, these analyses will enable us to address the adaptive process of *T. baccata* under several perspectives. From the molecular evolution angle, we aim to understand and compare evolutionary patterns for *T. baccata* and related species in Asia, the suggested diversification centre for the genus. From the angle of population adaptation, we expect to identify genes underlying adaptive differences, as well as increase our understanding on which types of genomic change favour adaptation to environmental changes. Candidate genes potentially responsive to environmental variation are now being selected throughout the transcriptome, and Single Nucleotide Polymorphisms (SNPs) within their sequence will be used as genetic markers to detect adaptation at the molecular level. As a further step, these thousands of SNPs will be genotyped in natural *T. baccata* European populations along environmental clines. Our long-term objectives are to characterize genetically the functional diversity of European yew natural populations and their potential for local adaptation confronted with impending ecological changes.

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Activation of retrotransposon-like sequences in *Pinus sylvestris* in response to stress conditions.

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Breeding of trees with increased phenotypic plasticity could also increase non-specific resistance to multiple abiotic or biotic stresses. Changes in environmental temperature causes pronounced effects on pest and disease distribution. Significant correlation in resistance to particular pests within half-sib families representing offspring with similar genotypes have been observed in Scots pine provenance trials (Jansons et al. 2012). This study examines retrotransposon-like sequences in the expression profile of stressed Scots pine trees. Retrotransposon activity can be a major factor in genome instability, rearrangements and therefore also plasticity of the genome and adaptation to changing environmental conditions.
Naturally occurring stresses such as heat shock and insect infection as well as treatment with specific chemicals like abscisic acid (ABA) and salicylic acid (SA) were tested. Two year old Scots pine ramets were used in this study to minimise genetic diversity between individuals. To discover transcriptionally active mobile genetic elements non-specific inter Primer Binding Site (iPBS) reactions were performed as described by Kalendar et al. (2010). Differentially expressed fragments amplified from stressed tree samples, but not from control tree samples, were excised, purified, and sequenced. Sequence analyses and mobile element identification was performed by searching within several nucleotide and protein databases. All fragments that were similar to known TEs in databases could be classified to at least an order or superfamily according to the classification proposed by Wicker et al. (2007).
Our first results suggested the existence of several groups of active retrotransposons in the Scots pine genome, which share differing similarity levels with known elements from other plant species (Voronova et al., 2011). Pine transcriptome analyses after treatment with plant hormones (salicylic acid and abscisic acid) revealed less retroelement-like sequences than after heat shock and insect damage. After SA treatment, only 5 retrotransposon-like sequences were identified with similarity with other retrotransposons at the nucleotide or protein level. After ABA treatment, only two retrotransposon-like sequences were found, both of which had been identified in other treatments. In comparison, after heat shock and insect damage 11 and 12 highly similar retrotransposon-like sequences respectively were identified.
The expressed retrotransposon-like sequences isolated after exposure to different stresses were often found to belong to different families of mobile elements. However, 63 sequences show no similarity to any annotated record in databases. Multiple sequence alignment of all unique fragments (125) identified in different studies reveals a complete difference between sequences expressed after heat shock and after application of plant hormones, as no fragments were shared between studies. Pine Woolly aphid caused damage activated only one retroelement-like sequence (Copia-2 TA-I) shared with heat shock ; one retroelement-like sequence and two unknown sequences are shared with SA treatment; and two unknown sequences are shared with ABA treatment. SA-ABA share one, abundantly expressed (in these studies) retrotransposon-like sequence (Copia 18 BD), and three unknown sequences. Specific primers to heat shock and insect damage response activated retrotransposon-like sequences were used to evaluate expression of retrotransposon-like sequences between samples subjected to various stresses. The amplification with specific primers shows cross amplification of some fragments. We assume that these

fragments are expressed in response to both stressors, but that the level of expression is different. Retrotransposon-like sequences are widely transcribed as many are also present in EST (Expressed Sequence Tag) databases of various plant species. Slight sequence variations were observed within a single isolated fragment, which could be explained by the origin of observed elements from multiple loci in the pine genome. Searches of nucleotide sequence databases reveals several elements that have characteristic structural features of full-length LTR (Long Terminal Repeat) retrotransposons in related species where more sequence information is available (*Pinus taeda, Pinus radiata*).

Nine specific LTR primers were designed using retrotransposon-like sequences with high similarity level to annotated elements obtained from heat shock and insect infestation experiments. Multiple band amplification with single primer on genomic DNA samples confirms presence of their multiple copies in Scots pine genome. It has not been clarified, how many copies from the identified retrotransposon-like sequences are activated in the complex pine genome, as translation of even a single element can cause transcription of many more non-autonomous retrotransposons with truncated sequences. Further studies of retrotransposon activation in pine could increase understanding of genomic rearrangements in response to stress conditions.

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Consistent ranking of *Populus nigra* genotypes for transpiration efficiency at leaf (^{13}C discrimination and leaf gas exchange) as well as at whole tree level under different vapour pressure deficits during growth.

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Poplar genotypes differ in transpiration efficiency (*TE*) at leaf level. Here, we test whether (i) whole plant *TE* is consistent with values at leaf level and (ii) vapour pressure deficit (*VPD*) during growth affects the genotype ranking for *TE* at leaf and whole tree level. Six *Populus nigra* genotypes were grown under two *VPD*. We recorded: (i) ^{13}C content in soluble sugars; (ii) ^{18}O enrichment in leaf water vs. source water; (iii) leaf-level gas exchange and (iv) biomass accumulation and water use by whole plants. Enhanced *VPD* had no effect on biomass accumulation but increased water-use which results in reduced whole plant *TE*. However, it increased intrinsic *TE* due to reduced stomatal conductance (g_s). It also resulted in an enhanced enrichment of leaf water and leaf matter in ^{18}O. Whole plant and intrinsic leaf *TE* differed significantly among genotypes and *VPD* had no effect on genotype ranking. g_s had a larger contribution to the genotype variability of intrinsic *TE* than *A*. Finally, ^{13}C composition of leaf sugars differed significantly from the modeled one and this difference was modulated by genotype and was smaller under high *VPD*. This may reflect changes in mesophyll conductance to CO_2 which may increase under high *VPD*.

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Is GxE interaction important in future proofing Sitka spruce breeding to climate change ?

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Background

The 700,000 hectares of Sitka spruce (*Picea sitchensis*, Bong, Carr.) in Britain are planted from relatively warm sites in Cornwall (51°N) up to the much cooler sites in northern Scotland (58°N). Genetic improvement of Sitka spruce in Britain started in the 1960s with the selection of superior individuals in plantation forests followed by genetic testing in comparative progeny trials. Today there is a re-selected breeding population of 240 individuals with superior growth rate and stem straightness and no decrease in wood quality. Forest managers have a choice of tested clonal seed orchards and vegetatively propagated material offering gains of around 25% for final rotation volume (Lee and Matthews; 2004) with 20% to 40% increases in quality sawlogs. Nearly 80% of the 32 million Sitka spruce trees planted in 2007 were from improved stock.

In spring 2005, three very large clonal trials were planted adjacent to each other on each of 3 climatically contrasting sites stretching the length of Britain (southern England; Wales; north Scotland); 9 experiments in total. The experiments are part of a Marker Aided Selection programme designed to link phenotypic traits with stable DNA-markers. Each experiment consists of a full-sibling family with no parentage over-lap between families. Each family is represented by 1,500 genotypes replicated 4 times at each site. The same 1,500 clones are present at all 3 sites. A standard control of unimproved material is randomly scattered through-out all the sites.

Objectives

To investigate and quantify the genotype by environment interaction (GxE) operating between the Sitka spruce clones across the three climatically contrasting sites and interpret any implications for applied tree breeding under an assumed changing (warmer) climate.

Methods

Trees in all 9 experiments were measured for 6-year height. Initial analysis was to investigate the mean performance of all the clones at each site against AT5 (cold site in north AT1300; warmer site AT1700 and warmest site in south AT2000 respectively) so demonstrating any evidence for a reaction norm. A uni-variate approach was then used to investigate stability parameters by employing the analytical software 'R' (2009). A downloadable 'R' package known as 'Agricolae' (2007) derived to investigate across sites stability of ryegrass, was used for the combined-site analysis at a clonal level. The 'Agricolae' package estimated across-site variance components including GxE and individual stability for all 1,500 genotypes within each of the three families. Clones were identified as highly unstable across the 3-sites (K8) through K4 and K2 down to K0 (highly stable across the 3-sites). In this way a global picture of GxE across the 1,500 genotypes within each family could be investigated at a clone-by-clone level.

Results

18,000 trees were measured for 6-year height at each site; 54,000 trees in total. GxE was found to be highly significant ($p < 0.01$) for each family. There were also highly significant differences between sites, and also clones within family ($p < 0.01$). The proportion of clones which are stable (K0) through to very unstable (K8) was similar across the three families being approx. 80% K0, 7% K2, 7% to 9% K4, and 5.5% K8. The indication is that 80% of the clones were stable across the 3 contrasting sites indicating little need for separate breeding populations. Selection of clones

based on a mean-performance across all sites should results in clones which are generally good at all sites from cold, to warmer, to warmest.

Conclusions

Although GxE may be expressed as significant at a population (family) level and approximately 6% of clones appear very unstable, there would seem to be significant superior clones expressing stability and adaptability across a temperature gradient such that they are good performers at all sites from cold to warmest. It was not possible to determine a repeated reaction norm between 6-year height and site warmth as measured by AT5. The evidence here would suggest that multiple selections or breeding populations for different climatic or edaphic conditions would not seem to be justified for Sitka spruce

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Phenotypic plasticity of growth-related traits in black poplar.

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Phenotypic plasticity is the ability of a single genotype to express more than one phenotype in response to heterogeneous environments. Elucidating the physiological underpinnings of phenotypic plasticity is of paramount importance in poplar as it allows the identification of genotypes that respond rapidly and adaptively to environmental conditions. Because biomass productivity depends upon the length of the growing season, the study of phenotypic plasticity of biomass production and growth-related traits such as bud set are important for the production of high-yielding genotypes.
Here, we present the most comprehensive analysis to date of variation of phenotypic plasticity of an F1 full-sib family (POP5) of *Populus nigra* under different environments.
The aim of this study is two-fold: (1) to evaluate genotype by environment interaction (G×E) for dry above ground biomass production and bud set; (2) to study plasticity of yield and phenology of POP5 genotypes among different sites.
Multiple regression equations linking above ground biomass with growth traits (circumference, height and number of sylleptic branches) were developed for each site and used to estimate the biomass production at individual level. Low values of broad-sense heritability (H^2) were observed for all biomass traits studied at each site (mean value of 0.19). The results also showed that some genotypes responded differently to changing environments, but the G×E interaction was not always significant. Moreover, some of the genotypes that demonstrated high biomass productivity with respect to the mean value of the full-sib family were among the least plastic ones the studied plantation sites. Using the new scoring system which consists of six crucial phenological stages of bud set process, our analyses highlighted that night length was the most important signal triggering the onset of growth cessation. Nevertheless, the effect of other environmental factors, such as temperature, increased during the process. Furthermore, a considerable role of G×E interaction was found in all phenological stages with the lowest temperature appearing to influence the sensitivity of the most plastic genotypes.

Keywords:
Populus nigra, G×E interaction, phenotypic plasticity, biomass production, bud set.

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Evaluation of early growth of provenance-progeny tests of Maritime pine across sites and implications for breeding in Spain.

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Maritime pine (*Pinus pinaster* Ait.) is a species of great economic and ecological importance in the western Mediterranean basin. We analysed the variation in growth traits (height, diameter) in multi-sites provenance-progeny trials established in northwestern Spain. In total, 398 open-pollinated families were evaluated, from 25 provenances (from Spain, France, Morocco and Portugal) at an age of 5.5 years. The analysis was conducted in two stages. In the first stage, a single site multi-trait analysis was conducted In the second stage of the analysis, sites were pooled to evaluate the importance of genotype x environment interaction, and the stability and adaptability of the genotypes, using Biplot analysis of genotype main effect model and the interaction genotype x environment (GGE). The best model in all locations included a first-order autoregressive structure through rows and columns to model residuals and a spatially independent error term. Individual heritabilities were moderate for the two growth characters (diameter h^2=0.09±0.08 to h^2=0.27±0.14; height h^2=0.12±0.07 to h^2=0.38±0.15). Genetic correlations between these variables were very high and positive in all trials (ranging between 0.91 and 1.0). The coefficient of population differentiation (Q_{ST}) was moderate to high (0.08±0.05 to 0.45±0.23 for diameter, and 0.17±0.09 to 0.43 ± 0.16 for height). The locality which showed a higher degree of divergence among provenances was (the locality F26MER (in the driest site). Provenances from Atlantic area perform better than the mediterranean. There is a significant GE interaction, and the GGE allow the identification of the macroenvironments to use within the breeding program. Based on the obtained results highlighted some important aspects were for future work with the species, and its proposed selection and mating strategies that would advance on the breeding program of the species.

Session 2: Genetic Basis of quantitative Traits

Invited Talk

Recent methodological advances in GWAS: multi-locus and multi-trait mixed-models.

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Genome Wide Association Studies (GWAS) are among the most popular approaches for investigating the genetics of complex traits. They usually consist in testing polymorphisms for association with traits in cohorts of individuals deemed to be unrelated. As long recognized, such design is subject to confounding by population structure, leading to an inflation of the test statistics and a high false positive rate. Inspired by Fisher's infinitesimal model and used for decades in breeding, mixed-modeling has more recently been proposed in the context of GWAS and is now becoming the default method to tackle population structure confounding in plants, animals and humans. However in its current implementation, the mixed-model performances are pretty limited because (i) it tests one polymorphism at a time which may not be appropriate for complex traits controlled by several large-effect loci, and (ii) it considers one phenotype at a time and thus does not take advantage of possible pleiotropy which can arise from multiple measurements on the same individuals. Motivated by this, we have proposed two extensions of the mixed-model, MLMM and MTMM that handle multiple locus and multiple traits respectively. With dedicated simulations, we have demonstrated that both approaches outperform existing methods in terms of power and false discovery rate. As a result, when applied to real human and Arabidopsis thaliana data, we identified new associations and especially cases of allelic heterogeneity with MLMM and pleiotropy with MTMM. We also applied MTMM to multiple measurements of flowering time in A. thaliana across several environmental conditions and identified a locus whose effect depends on the environment. Both approaches are efficiently implemented, making the analysis of large datasets practicable.

Oral presentations

QTL approach

The genetics of water-use efficiency in maritime pine and its relation to growth.

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In the context of global climate change, developing genotypes for water limited conditions is becoming a mandatory selection criterion of the maritime pine breeding program. The selection of trees maintaining higher assimilation rate and growth and / or saving water is crucial. Water use efficiency (WUE) estimated with $\delta^{13}C$ is an integrative trait usually used for such objective. The present work provides original data about the genetics of WUE and its relation to growth in this ecologically and economical important species. First, genetic components (heritability, variance components, correlations with growth related traits) were estimated in a wide range of genotypes from a progeny trial (50 half-sib families) planted in three different sites characterized by different water status. Secondly, the genetic architecture of WUE was studied in a unique F2 pedigree (selfed seeds obtained from a single hybrid tree derived from a controlled cross between Landes and Corsican genotypes) for $\delta^{13}C$ and ring width at two different ages (2 and 10 years old) corresponding also to two different environmental settings: a pot experiment in greenhouse (200 genotypes) for the former and a field trial (531 genotypes) for the later. $\delta^{13}C$ heritability ranged from 0.24 to 0.41 in the three sites and was higher than that of growth. Several QTLs for $\delta^{13}C$ were detected confirming the polygenic determinism of WUE. The main finding of this study is the detection on linkage group #12 of a major QTL (more than 60% of phenotypic variance explained) detected in the field experiment and also found in the pot experiment (although less strong, 7.7% of variance explained) on the same genomic location. This work also showed that at this QTL position, the allele from Corsican grandparent is involved in a higher WUE is recessive in comparison to the Landes allele. These results are discussed in relation to growth related traits and in the context of the breeding program for maritime pine.

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QTL mapping and genomic characterization of phenology in black poplar

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The genetic control of important adaptive traits, such as phenology, is still poorly understood in most forest trees species. Poplar is an ideal model tree to study phenology because of its indeterminate shoot growth. Thus, a full-sib family derived from an intraspecific cross of P. nigra with 162 clonally replicated progeny was used to assess genetic variation of bud set in two sites of contrasting environmental conditions.
Six crucial phenological stages of bud set were scored. Night length appeared to be the most important signal triggering the onset of growth cessation. Nevertheless, the effect of other environmental factors, such as temperature, increased during the process. Descriptors of growth cessation and bud onset explained the largest part of phenotypic variation of the entire process. Quantitative trait loci (QTL) for these traits were detected. For the four selected traits (the onset of growth cessation (date2.5), the transition from shoot to bud (date1.5), the duration of bud formation (subproc1) and bud maturation (subproc2)) eight and sixteen QTL were mapped on the maternal and paternal map, respectively. The identified QTL, each one characterized by small or modest effect, highlighted the complex nature of traits involved in bud set process. Comparison between map location of QTL and P. trichocarpa genome sequence allowed the identification of 13 gene models, 67 bud set-related expressional and six functional candidate genes (CGs). These CGs are functionally related to relevant biological processes, environmental sensing, signaling, and cell growth and development. Some strong QTL had no obvious CGs, and hold great promise to identify unknown genes that affect bud set.
This study provides a better understanding of the physiological and genetic dissection of bud set in poplar. The putative QTL identified will be tested for associations in *P. nigra* natural populations. The identified QTL and CGs will also serve as useful targets for poplar breeding.

Keywords:
Bud set, Populus nigra, quantitative trait loci, candidate genes, seasonal growth cycle.

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QTL mapping and transcriptome sequencing to identify candidate genes for drought tolerance in *Salix*.

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Salix species are good candidates for short rotation coppice cultivation due to their fast growth and ease of vegetative propagation. Their high genetic diversity (Berlin *et al*. 2011) and short generation times also makes them suitable for breeding. New cultivars adapted to a drier climate and for areas where water can be a limitation are needed for a sustainable and high biomass production of *Salix* in Europe. Variation in response to drought has been shown both within and between species and species hybrids of *Salix* (Weih & Nordh, 2002; Bonosi *et al*., 2010). To develop *Salix* varieties better adapted to drought conditions we need to understand the genetic architecture of drought, reveal genomic areas underlying variation in drought tolerance and find candidate genes responsible for the trait variation. In order to detect genomic areas and identify candidate genes two experiments were conducted; a quantitative trait loci (QTL) study in a greenhouse during summer 2009 and a drought experiment for transcriptome sequencing in a phytotron during spring 2011.

The first experiment included parental clones and 198 randomly selected genotypes from the mapping population S_1 (Berlin *et al*. 2010). A complete randomized block design with three treatment combinations of nutrient and water availability was replicated three times in the experiment. After 20 days of treatment the plants were harvested; leaves and shoots for each plant separated, dried and weighted. In addition, leaf area, chlorophyll content (SPAD) and dry weight was measured on three sample leaves of each plant to enable analysis of growth and nitrogen content. The genetic linkage map for the S_1 population is based on SNP, microsatellite and AFLP markers and the consensus linkage map consists of 495 markers and has a length of 2477 cM (Berlin *et al.* 2010). The SNP markers were developed by using the sequenced poplar genome as reference and enabled an alignment of the linkage map to the *Populus* physical map. QTL analyses were performed on trait mean values for each genotype and treatment combination using interval mapping and a maximum likelihood model. A LOD threshold value to determine significant QTL was estimated with a permutation test of 5000 repetitions. One and two LOD confidence intervals for each QTL were estimated.

For the second experiment two sibling clones (# 520 and 592) from the mapping population S_1 showing differential response to drought were selected. A drought experiment was conducted in a phytotron using 15 plant of each genotype in a drought treatment with permanent water stress for 31 days and 15 plants in well-watered conditions. The water stressed plants were kept near the wilting point and received approximately 50 % of the water supplied compared to the well-watered control plants. Two harvests were carried out during the experiment, one initial control harvest before the start of the treatment period and a final harvest at the end of the experiment. The harvested plants were separated into different growth parts, dried and weighted. Total leaf area was measured for each plant at harvest and SPAD measurements repeatedly taken during the treatment. Changes in plant growth and N economy over time were analysed using growth analysis based on biomass and leaf area changes between the two consecutive harvests. The day before the final harvest leaf and root tissue samples were collected and total RNA was extracted.

The RNA samples were sequenced using Illumina HiSeq 2000 and the sequences were mapped to the *Populus trichocarpa* genome. To assess a drought response in gene expression, the "raw counts" (number of mapped reads to every mRNA feature in the reference genome) for drought treated plants were compared with their respective controls within tissues and genotypes. Four pairwise tests were thus constructed: 592-leaves, 520-leaves, 592-roots and 520-roots. To identify candidate genes for drought response the significant genes in these comparisons with a ≥10-fold difference were selected and checked if genomic location corresponded to a QTL area. Information regarding gene function was retrieved by comparing *Populus* protein sequences towards the protein sequence in *Arabidopsis thaliana*.

Growth and nitrogen analysis of the phenotypic data showed that nitrogen uptake and nitrogen efficiency are important traits for improvement of growth under drought (Weih et al. 2011). Higher leaf nitrogen content was found in drought tolerant genotypes compared to drought sensitive genotypes. In total 60 QTLs were identified for growth and nitrogen traits; 23 were related to biomass and 37 to nitrogen traits. Most of the nitrogen traits mapped to linkage groups (LGs) II, VI, XIII and B. At LG VI, QTLs for SPAD as well as QTLs for mass based leaf nitrogen content identified in all treatments mapped to the same position. Colocating QTLs for nitrogen use efficiency in different treatments mapped at LG XIII. The phenotypic variation explained by each QTL varied from 7.7 to 41.9 % of the total variance. The 18 QTLs explaining most of the variation were in 17 cases nitrogen related traits. QTLs for biomass traits in general explained around 10 % of the variation. Drought candidate genes were found in all QTL areas and they included genes known to influence drought tolerance as DREB and heat stress transcription factors, gene responding to water deprivation and genes responsive to abscisic acid. The results are encouraging with respect to future development of marker-assisted breeding. Further studies *e.g.* association mapping and qPCR to verify the effect of specific candidate genes, are needed.

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Association genetics approach

Genetic association for growth and biomass in maritime pine.

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Genetic association, that is the establishment of statistical correlations between quantitative traits and marker genotypes, can provide valuable information for breeding, including insights on the genetic architecture of traits and on those functional markers amenable to be used in molecular breeding approaches. In this study, we used mixed models and ridge regression (that accounts for lack of independence of markers) to build single- and multi-locus models of genetic association between 302 SNPs and a wide range of growth and biomass traits measured in two common gardens, one located in France (Pierroton: humid site) and the other in Spain (Cálcena: dry site). Neutral molecular markers (nuSSRs and control SNPs) were used to correct for population structure in genetic association analyses. Alternatively, BLUPs that exclude the population effects were also computed. Significance values were obtained using a permutation procedure that substantially reduced false positives and were further corrected by multiple testing using a False Discovery Rate (FDR) approach. Correlation between observed and predicted breeding values based on different SNP combinations and ridge regression (RR-GLM) gave information on multi-locus associations (see Resende *et al*. 2012 for a comparative of genomic selection methods applied to pine). Several associations were detected in the humid site, after removing potential false positives, normally involving SNPs from four strong candidate genes: *KOR* (*KORRIGAN* encodes for a β 1-4 endo-glucanase; see Pot *et al*. 2005), *lp3-1* and *lp3-3* (that are two member of the ASR small gene family that have previously been suggested to have a role in drought response) and *CAD* (that encodes for a key enzyme which catalyzes the last step of *lignin* monomer biosynthesis), and total height, diameter, polycyclic growth and total biomass. For example, SNPs in *KOR* (several linked ones) and in lp3-1/lp3-3 (combined) genes explained around 8% each of the phenotypic variance for total height in the humid site. SNPs in *KOR* appeared also to be responsible for about 15% of the variance in polycyclic growth in this trial. Finally, the combined effects of SNPs from the four candidate genes acting together were able to explain c. 15% and c. 4% of the variance in diameter and total biomass, respectively. These SNPs, in particular those in *KOR*, are expected to contribute to current breeding efforts in maritime pine.

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Basis of genetic variation

A large portion of the small RNA population of *Picea abies* is derived from NBS-LRR resistance genes – a novel mechanism in pathogen defence?

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Small RNA (sRNA) including miRNA and siRNA are important in the regulation of diverse biological processes. Comparative studies of sRNA from plants have mainly focused on miRNA even though they constitute a mere fraction of the total sRNA diversity. In the present study we report results from an in-depth analysis of the sRNA population from the conifer *Picea abies* and compared the results to those of a range of species from the plant kingdom. Combining the results of different prediction programs we could validate most of the already characterized miRNA families from spruce, but also detect 20 novel ones. The vast majority of sRNA sequences in *P. abies* is however not miRNA sequences and can rather be assigned to 21 nucleotides long siRNA, of which a large fraction originate from degradation of transcribed sequences related to NBS-LRR (Nucleotide Binding Site-Leucine Rich Repeats) type resistance genes. Over 90% of all genes predicted to contain either a TIR or an NBS domain showed evidence of siRNA degradation. Data further suggests that those phased siRNA are initiated from miRNA guided cleavage, often by an abundant 22 nt miRNA. Comparative analysis over a range of divergent plant species revealed a large variation between species in the abundance of this phenomenon. The process seemed to be virtually absent in several species, including *Arabidopsis thaliana*, *Oryza sativa* and non-vascular plants, while particularly high frequencies were observed in *Vitis vinifera* and *Populus thrichocarpa*. This divergent pattern between species might reflect a mechanism to limit runaway transcription of these genes in species with rapidly expanding NBS-LRR gene families. Alternatively it might reflect variation in a counter-counter defence mechanism partly affected by differences in life history traits, *e.g.* perennial versus annual life cycles. A major difference between annuals and perennials, affecting the evolution of resistance, is that perennials will with almost certainty encounter many different pathogens before reproduction, and that the long generation times confers problems in matching the evolutionary rates of the pathogens.

Differential expression in a complex poplar hybrid pedigree via RNAseq analysis.

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Heterosis measures the deviation of performance of the hybrid offspring compared to the closer extreme parent, often leading to desired phenotypes such as increased biomass production. Several hypotheses have been proposed to explain heterosis at a molecular level. A common point in explaining the hybrid vigour is the increase in heterozygosity in hybrid offspring compared to their parents. Complementation of deleterious alleles via dominance or pseudo-overdominance, and allelic interactions (overdominance) are some of the proposed mechanisms. Although many researchers refer to such mechanisms to single nucleotide polymorphisms, they apply to different phenomena as well. An intriguing hypothesis is that the two parental species may show different expression patterns, maybe as a consequence of polymorphisms in regulatory regions. In case of cis-regulatory variation, hybrids will inherit two alleles, each showing an expression pattern similar to the pattern of the allele of origin, and this may contribute to hybrid vigour. Thus, genes with different expression in the two parent species may as well be candidate markers of heterosis.
We performed an RNAseq experiment on a complex poplar pedigree consisting of two *P. nigra* male parents, two *P. deltoides* female parents and four hybrid offspring.
We identified 1685 genes differentially expressed between *P. nigra* and *P. deltoides* (nominal p-value < 10^{-10}). Of them, 897 had higher expression in *P. nigra* and 788 in *P.deltoides*. In addition, we identified 514 genes differentially expressed in the offspring compared to midparent value (nominal p-value < 10^{-10}). Of them 305 had higher value in offspring and 209 had higher midparent value.
Given the limited sample size the differentially expressed genes are to be considered as potential markers of heterosis and their involvement needs to be confirmed by additional follow up studies.

Keywords:
RNAseq, heterosis, poplar, pedigree

Epigenetic adjustment of phenology in *Picea abies*. Does it matter to tree breeding?

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Epigenetics is now recognized as a real phenomenon that significantly influences many traits in plants and other organisms. In Norway spruce changes in phenology, that we now consider to be due to epigenetics, was first described by Bjørnstad (1981) who showed that progeny of provenances from Northern Norway that had been moved to a seed orchard in Southern Norway, had delayed bud set compared to progeny produced in Northern Norway from the same families. However, Engler, cited by Langlet (1971), had already in 1913 observed that lowland spruce cultivated at high altitude produced a progeny which almost corresponded to plants of the local high altitude provenances. These results are very similar to what was observed in progeny from German provenances in Norway (Dietrichson 1964, Skrøppa *et al*. 2010).
By use somatic embryogenesis Kvaalen and Johnsen (2009) showed that the temperature during embryo development causes a very large shift in bud set and elongation growth in young plants, and proved that the shift is not due to pollen selection or maternal effects. The somatic plants where planted in the field in 2007. Here we present results from assessments of phenology and height growth to show that the differences observed in young plants persists. In one clone, ramets from cold embryogenesis (18 C) the average date of bud burst was 8 of May in 2011, whereas in ramets from warm embryogenesis (28 C) the corresponding date was 28 of May. Thus the data show that bud burst, a trait that has showed high heritability in numerous studies, is also under very strong control of an epigenetic memory that responds to temperature during embryogenesis.
Because the effects on phenology are lasting, and phenology is of great importance to plantation establishments in the Nordic climate, epigenetics should be taken into consideration when seed orchards are established and where the plant material is to be used.

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Posters

QTL mapping of rust resistance genes in *Salix* and identification of candidate genes.

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Salix cultivars used for biomass have the advantage of rapid growth, accessibility to clonal propagation by means of cuttings, high energy content, and the ability to act as a biofilter of contaminants. One major drawback with large-scale production using clonal material is the risk of building up fungal pathogen populations that may overcome or break the host resistance to disease. This is particularly risky when basing any resistance trait on a small number of major gene components. The willow leaf rust fungus, *Melampsora larici-epitea*, can cause large production losses in biomass willow as a result of reduced photosynthesis and premature defoliation. A high rust resistance is therefore one of the most important breeding goals. It would be preferable to build up rust resistance that is based on several minor genes (i.e. quantitative resistance) and hence more complicated for the fungus to adapt to. The aim of this work is to characterize resistance genes in the current breeding material and develop molecular markers for a variety of resistance genes to be used in marker-assisted breeding.
For mapping of resistance genes we are utilizing two linkage maps of *Salix*, one based on a hybrid backcross between *S. viminalis* and *S. schwerinii*, and the other based on a pure *S. viminalis* family. By doing controlled infection experiments in growth chambers we were able to characterize various resistance reactions in these families. Measurements were made of latent period, number and size of rust pustules, and necrotic flecking. We have also done assessments of rust resistance in the field on the same individuals. By doing QTL mapping (mapping of quantitative trait loci) several genomic regions that are important for rust resistance have been identified in the two populations (Samils *et al*. 2011).
We constructed BAC libraries containing the genomes of the parents of the hybrid population in order to investigate the presence of specific rust resistance genes in the QTL regions. A three-step PCR screening procedure, based on the BAC library pool system, was performed to identify genomic fragments around the resistance QTLs by use of the map markers. Positive BAC clones were sequenced by high-throughput Illumina sequencing. In the genomic region of a major QTL we could identify two candidate genes with high similarity to annotated rust resistance genes in the *Populus* genome. Specific primers were developed for these genes to study their sequences in a larger material. We have identified SNPs (single nucleotide polymorphisms) that can be used to track the different alleles and thus follow the inheritance of the alleles in a pedigree.
Our results show that rust resistance genes can be identified with the current approach. Further work is needed to verify the candidate resistance genes (e.g. by gene expression analysis), and additional QTL regions need to be investigated to identify a broader range of rust resistance genes.

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What do we know about the genetic determinism of cavitation resistance in maritime pine?

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Resistance to cavitation is the main determinant of tree survival under severe drought. The interspecific variation of this trait is well known, but data at intraspecific level remains embryonic. Quantifying genetic variation and phenotypic plasticity is therefore essential to estimate the adaptive potential of perennial organisms in the context of climate change. Combining for the first time the *in situ* characterization of natural populations with common garden experiments in xeric and mesic sites, we estimated variance components (genetic G, environmental E, and interaction GxE) of resistance to cavitation based on 506 genotypes of *Pinus pinaster*.
Resistance to cavitation displayed low phenotypic (CV_P<7%) and genetic (CV_A=4.9%) variances, limited phenotypic plasticity and a high heritability compared to other traits such as growth. Q_{ST} was significantly lower than F_{ST}, indicating an evolutionary stasis for P_{50}, rather than genetic drift. This pattern of variation strongly suggests that resistance to cavitation is buffered by genetic and, to a lesser extent, environmental variations in pine species.

Keywods:
Hydraulic adjustment, drought tolerance, cavitation resistance, provenance-progeny trial, Pinus pinaster, phenotypic plasticity, genetic variation, Q_{ST}/F_{ST} comparison.

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SNP discovery from candidate genes and *de novo* NGS transcriptome sequencing in Aleppo pine.

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A central challenge in evolutionary biology and breeding is to identify genes underlying ecologically- and economically-important traits and to describe the fitness and productivity consequences of naturally occurring variation at these loci. Advances in sequencing technology (the so-called Next-Generation Sequencing or NGS) have allowed obtaining full- or nearly-full transcriptome sequences in non-model species at an affordable cost. NGS-based SNP (Single Nucleotide Polymorphisms, or point mutations, referred to single base changes in DNA) discovery is very challenging in species without a reference genome due to poor alignment of short sequence reads of different individuals generated by current NGS technologies. In this work, we present the *de novo* design of a 384plex SNP-genotyping assay in *Pinus halepensis* for further development of multiple applications, from genetic association mapping to the study of demographical history in this non-model species. The 384 SNPs used for the assay were obtained from two sources: i) 161 amplicons sequenced within the framework of different international projects, such as EvolTree (Conifer Re-sequencing Initiative for European Conifers, CRIEC) and CRSP (Conifer Re-Sequencing in Pinaceae); and ii) *de novo* NGS transcriptome data from two individuals with contrasting fire-related phenotypes.
The traditional Sanger sequencing (161 amplicons) approach yielded 625 high-quality SNPs (1 SNP every ~116 bp of *Pinus halepensis* population sequence data), many of them narrowly restricted to the eastern Mediterranean populations. Then, starting with the 625 good quality SNPs and through eye-check validation, we applied filters to remove SNPs too close from each other and those in strong linkage disequilibrium (LD), except in cases where the candidate gene was particularly interesting (e.g. a strong candidate for drought response), resulting in a final set of only 144 usable (with current Illumina genotyping technology) SNPs. As most of the SNPs identified were in strong LD, probably due to a demographic history characterized by founder effects during range expansion (Grivet *et al.* 2009), usable SNPs were very often reduced to just one SNP per gene. The second set of 240 SNPs was selected from a transcriptome unigene set obtained through Illumina HiSeq 2000 sequencing of two *Pinus halepensis* individuals with contrasting fire phenotypes. Transcriptome sequencing produced *c.* 60,000 unigenes. From those, we applied sequential quality filters (e.g. contig length and coverage) to select *c.* 18,500 polymorphic SNPs from 3,000 unigenes. This set was enriched for SNPs that i) were fixed for different alleles in the two trees, ii) belonged to genes with differential expression across individuals (estimated by different RPKM, i.e. reads per kilobase per million mapped reads) and iii) belonged to genes with elevated values of non-synonymous to synonymous divergence (using spruce as outgroup). Finally, using an annotation obtained by BLASTx, we reduced the number to *c.* 3,050 polymorphic SNPs from 427 unigene contigs and used a NGS assembly visualization software (Tablet) to eye-check and validate the selected SNPs, yielding a total number of 240 usable SNPs for genotyping. All 384 SNPs (144 from Sanger amplicons and 240 from NGS transcriptomes) had score 1 (high potential success rate) when evaluated by the Illumina Assay Design Tool. Genotyping of about 2,000 trees, including two genetic association populations and natural populations with conservation interest, with this array is underway.

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Flowering Locus T / *Terminal Flower Like* 2 (*FTL2*) gene is involved in the control of growth rhythm in Scots pine (*Pinus sylvestris*).

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Perennial plants such as conifers adapt their growth rhythm to seasonal changes in the environment. Growth cessation, bud set and dormancy are among the main adaptations used by the plants to prepare for winter. These adaptive traits are mainly controlled by photoperiod, temperature and light quality and show a high correlation with clinal variation in different species including Scots pine [1,2,3,4]. In conifers, efforts have been made to understand the genetic basis of these traits. A Norway spruce's *Flowering Locus T* / *Terminal Flower Like* gene (*PaTLF2*) has been shown to be a key gene mediating a photoperiodic control of annual growth rhythm[5,6]. However the photoperiod reactions of Scots pine can be different from those of Norway spruce[7]. To examine whether the homolog gene in Scots pine (*PsTFL2*) has an equivalent function, we have studied the expression of the gene in three different populations of the species (Northern and Southern Finland and Poland).

Seedlings of eight families from each population were grown in a greenhouse under natural photoperiod conditions and sampled every two weeks until bud set. To investigate how the expression of the different genes would vary under artificial photoperiod compared to the natural photoperiod, seedlings were grown in growth chambers under constant light for two and a half months before transfer to three different photoperiod conditions (20 h/4 h, 17 h/7 h and 8 h/16 h light/dark) in growth cabinets and sampled every 4 hours during 4 days. Seedlings were measured regularly to detect growth cessation and subsequently, visible buds were scored. Gene expression of *PsTFL2* was studied by RT-PCR.

There was a significant increase in the expression of the gene preceding a boost of bud set and this expression decreased after a high level of bud set in each population. Under artificial conditions, the expression of *PsTFL2* showed an overall increase following the transfer from continuous light to short day conditions, with a higher expression in the northern populations. Furthermore, there were a correlation between the length of the dark period and the proportion of plants setting bud after transferring the plants back to continuous light.

Our gene expression results showed that, like in Norway spruce, *PsFTL2* seems to be a key gene in the control of bud set in Scots pine, and the pattern of this expression is different between populations from different latitudes.

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Growth behaviour and epigenetic markers in Norway spruce.

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Forests provide the renewable sources for a wide variety of industries like timber, pulp and paper, furniture and bioenergy/biofuel industries. For some of these areas Norway spruce is providing the prime source for various applications. Molecular markers will serve as tools to select trees and planting material for sustainable future forests, which are able to deal with the challenges of predicted future climate change. Besides DNA encoded features, there is also the level of epigenetic modifications which influences a plant's reaction to the environment.
For our studies on epigenetic variation we chose individuals varying in flushing behaviour, which was assessed over 3 subsequent years in 3 putatively autochthonous Austrian populations of Norway spruce along an altitudinal gradient. Out of 450 individuals, DNA of individuals exhibiting consistent extreme phenotypes (i.e. early/late flushing) was pooled. DNA pools were subject to a methylation filtration technique, the generated fragment-libraries subjected to 454 NGS analysis. *In silico* comparison of DNA fragments represented in the two different filtration pools revealed fragments 56 candidate regions (clusters of more than 6 reads) putatively differentially methylated in early and late flushing trees respectively. Out of these regions, 12 annotated as organellar elements and were discarded from further analysis. Differences in methylation patterns were validated by bisulphite sequencing analysis in the 3 selected regions showing high CG content. A set of 57 trees from 3 different altitudes were tested with MSP (methylation sensitive PCR) amplification based on the result of the bisulphite sequencing. Results showed different degrees of methylation in early and late flushing trees as well as differences in trees from different elevations.

Identification, characterization and comparative analysis of repetitive sequences in conifers.

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Conifers are the most widely distributed group of gymnosperms in the world. They have large genome size (ranging from ~6,5 Gb to ~37 Gb) compared with most animal and plant species. They are wind-pollinated, highly heterozygous, and long-lived trees with vegetative phases extending from one to several decades. Conifers are both economically and ecologically important. Indeed, they are the most important plant group of wood and fiber crops and are dominant members of many ecosystems, primarily in boreal forests. Since the conifers are so important, a better understanding of their genome is required. The Spruce Genome Project aims to sequence the Norway spruce (*Picea abies*) genome. The availability of its genome sequence will provide a valuable resource for a better understanding of conifer genome structure and evolution. Furthermore it will spark research to generate new tools for molecular breeding aimed at improving tree quality and productivity. The sequencing of a conifer genome has to cope with two main problems: the huge size of the genome (approximately 20 Gb in *P. abies*) and its complex structure due to the highly repetitive sequence content. About 75% of the conifer genome consists of repetitive DNA sequences, while the remaining part of the genome contains unique and low copy sequences. A high proportion of the unique and low copy sequences are likely transposable element related sequences that have become highly diverged in the course of evolution.

Within the framework of the Spruce Genome Project, our work focuses on the genome repetitive fraction of six conifers (*P. abies*, *Abies sibirica*, *Juniperus*, *Pinus sylvestris*, *Picea glauca*, *Taxus baccata)* and an outgroup (*Gnetum*). Starting from a set of *P. abies* data including 100000 454 reads having length greater than 700 bp, the current assembly and 4 completely sequenced BACs, we *de novo* identified and characterized repeats in using an *ad hoc* pipeline relying on the software RepeatScout, cap3 and cd-hit. We then manually crosscheck the output of LTR-finder software run on contigs larger than 10 Kbp. In doing so we isolated 126 complete LTR retroelements (LTR-RTs) bearing clear target site duplications (TSDs). All the identified repeats were characterized using similarity searches against public and in house database of transposable elements. Our repeat library includes 1773 sequences: 764 LTRs, 25 LINEs, 23 DNA-TEs and 961 unclassified repeats. When the current assembly was masked using RepeatMasker fed with the isolated repeats, around 70% of the sequence resulted repetitive. The main category of the repetitive fraction is constituted by LTR-RTs (83.9%). Using the same approach with a random sample of 100000 454 reads, we characterized the repeat abundance in the other species. LTR-RT elements are the most represented TEs across all the different genera.

All the isolated LTR-RTs in the seven different species underwent phylogenetic analyses. Paralogous copies of a 100 aminoacid residues from the reverse transcriptase coding domain were isolated and aligned and eventually used to build Neighbor-Joining phylogenetic trees. The complement of LTR-RT elements is ancient for both Ty1-copia and Ty3-gypsy superfamilies: most of the identified families include paralogous from all the conifer *genera*. Some families include

paralogous from the outgroup (*Gnetum*) too. However the overall majority of the retrotransposition events took place after the *genera* separates: the internal highly bootstrap supported clades are mostly separated according to different *genera*. For both superfamilies there are cases showing the occurrence of sustained species-specific activity of certain LTR-RT families. A striking example took place in *Juniper* where a single Ty1-copia family accounts for the vast majority of the LTR-RT elements of this species. A similar, although less dramatic example, has been seen for Ty3-gypsy elements in *Gnetum* where just two families comprise the overall majority of the Ty3-gypsy elements identified in the species. The availability of the pair *P. abies*/*P. glauca* allowed us to study the LTR-RT retrotransposition dynamics in the frame of a more limited evolutionary time. The vast majority of the retrotransposition activity seems to have taken place before speciation since the LTR-RT paralogous from both species consistently mix together in strongly bootstrap supported clades. Some TEs show a quite high sequence conservation at nucleotide level across genera separated by large evolutionary distances: data supporting this aspect will be discussed.
We also identified microsatellites in the repetitive and not repetitive fractions of all the seven species studied. Microsatellites are mainly associated with non-repetitive DNA. Moreover, we identified all the tandem repeats having a repeat unit longer than 25 bp: 10 satellite sequences were isolated in *P. abies*. Eight of them are shared with *P. glauca*, the remaining two are unique to *P. abies*. No one is shared with *Juniperus*, *Abies*, *Taxus, Pinus* and *Gnetum*.

Keywords:
Repetitive sequences, retrotransposon, conifers, evolution

De novo assembly in *Populus nigra* to lead comparative genomics analysis applied to breeding

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The analysis of variation in forest trees has revealed that their genomes are characterized by high levels of nucleotide polymorphism and structural variation, consisting of insertion/deletions of small size, mostly due to recent insertions of transposable elements, and very large size termed Copy Number Variants. These observations indicate that a single genome sequence might not reflect the entire genomic complement of a species, and prompted researchers to introduce the concept of the plant pan-genome, which includes core genomic features common to the species and a dispensable genome composed of non-shared DNA elements that can be individual- or population-specific. The pan-genome concept can be extended also to closely-related species, as in the *Populus* genus where a number of species are inter-fertile. The interest in characterizing the dispensable genome rely on the key role that the non-shared DNA elements might have in breeding situations like the phenotypic plasticity, the phenotypic variation and the heterotic behavior.
De novo sequencing of a genome is today accessible and affordable thanks to the advent of the next-generation sequencing technology that has made sequence data production accurate, cheap and fast. Since the release of this new technology, many genome sequences have been published but comparative and structural genomics analyses are still a challenging issue. Given the huge amount of sequence data available, nowadays a special effort has to be given to the latter analyses which are crucial to better understand both the evolution and the composition of the different genomes.
Exploiting the Illumina technology and a de novo assembly approach, we obtained the genome sequence of an Italian genotype of *Populus nigra*, a native European poplar species of importance for the wood and paper industry, and thus a key species in different breeding programs. We sequenced the heterozygous poplar genotype at high coverage (~80X) using different types of libraries in order to solve repetitions and allow the contig scaffolding: technical and critical aspects will be discussed. We focused on three different software to perform the assembly: results and comparisons will be provided considering the reconstruction of the genome with a particular focus on the heterozygous portions and on the gene content.
On the selected assembly (length 319 Mb and N50 size 6130 bp), we developed a comparative genomic analysis pipeline to characterize the assembled sequences in terms of gene potential and repetitive elements, and similarity to *P. trichocarpa*, the American poplar species sequenced using the Sanger method and known to be inter-fertile to other poplar species. A test experiment was run on almost 200 randomly-selected contigs in order to validate the pipeline which, due to the low error rate detected, proved to be efficient and accurate in comparing and characterizing the genome sequences concerned. We propose our pipeline as a comparative genome tool to highlight interesting areas of the genome of different closely related individuals or organisms to be selected for breeding purposes.

Keywords: *De novo* assembly, *Populus nigra*, Illumina technology, pan-genome

Establishment of a composite SNP linkage map for maritime pine

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Offspring of three-generation outbred and inbred mapping pedigrees were genotyped using a 12k SNP infinium array. The inbred pedigree consisted of a classical F2 population resulting from the selfing of a single inter-provenance (Corsica x Landes) hydrid tree, while the outbred pedigree (G2) resulted from the controlled cross of two intra-provenance (Landes x Landes) hybrid trees. As a result, three linkage maps were generated based on SNP markers, one from the parental genotype of the F2 (1,121 framework markers in 1,708 cM), two for both parental genotypes of the G2 (549 and 618 framework markers in 1,447 and 1,425 cM for the female and the male parent, respectively). A composite linkage map was established by combining the three component maps F2, G2F and G2M. In comparison to consensus maps that are computed from genotypic data of multiple populations, composite maps are directly obtained from individual linkage maps. We used two approaches that are based on the graph theory: MergeMap (http://mergemap.org) and LPmerge (this study). The LPmerge algorithm uses linear programming (LP) to generate a consensus map with minimum error relative to the component linkage maps. LPmerge was found to perform better than the former. The resulting composite map (1,838 SNPs in 1,712 cM) is now being used to study the landscape of genetic diversity and linkage disequilibrium along the linkage map.

Session 3: Re-thinking forest tree breeding for optimality

Invited Talk

Moving Towards the Fourth Cycle of Loblolly Pine Breeding in the Southern United States

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The economic incentive to improve progress in tree breeding is strong for the southeastern US. Increasing the genetic gain per year by only a fraction of one percent has regional financial benefits of millions of dollars. We are developing the fourth-cycle breeding strategy for the North Carolina State University Cooperative Tree Improvement Program to meet challenges brought by climate change, while enhancing genetic gain and maintaining long-term genetic diversity. Algorithms used in animal breeding programs are being explored to illustrate the tradeoffs between gain and average inbreeding level for multiple generations. Pedigree analysis tools were used to provide insights for our population management options. Our preliminary pedigree analysis shows that there is minimal inbreeding in our third-cycle Coastal population, the largest of the three regional populations. The population has an effective number of founders of 265. Multi-generation simulations show that a breeding population with a census number of 200 individuals (with effective number of founders =149) can produce gain for at least five generations. We are also developing strategies to incorporate DNA markers for pedigree reconstruction and genomic selection. These approaches will serve to increase genetic gains by reducing the time and effort required for progeny testing. Historical weather records and provenance growth data are also being analyzed to determine the universal response functions of different seed sources and genotypes for adaptive traits such as cold hardiness, heat tolerance, and drought stress, for incorporation into deployment strategies based on scenarios of future climate alternatives.

Oral presentations

Evaluating alternative breeding strategies.

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Forestry in Europe spans the entire spectrum from low-intensity to intensive forest management. In addition to the choice of tree species and silvicultural methods, intensive forestry most often uses tree improvement to further adapt trees to environmental conditions, as well as to develop economically important tree characteristics. This paper discusses best practice for evaluating and fine-tuning breeding strategies, respecting tree-species characteristics and breeding objectives. Since the main emphasis is on intensive breeding for plantation forests supplying wood-based industries, we focus on evaluation of six alternative strategies representing intensive tree breeding.

The term "breeding strategy" relates to a plan for managing populations to meet the goals of a breeding programme. The goals set for breeding can be exceedingly diverse and can include economic traits, adaptability and gene conservation. The context in which breeding is carried out is considered under four groupings of important factors: (1) Biological; (2) Economic; (3) Institutional; and (4) Socio-political. Many of these factors will impose serious constraints on what can be accomplished and its degree of difficulty. An effective breeding strategy is not possible without a good understanding and realistic assessment of these factors.

The development of a breeding plan usually starts with an assessment of the regeneration systems anticipated for the species. The gains from tree breeding can only be realised when bred material is deployed to the forest, be it by planting or direct seeding. The next task is assembly of a breeding population (BP). For all but the smallest programmes, a closed BP is usually assembled from plus-trees selected in natural populations, and managed to generate recruitment populations. These may be generated through low-tech approaches involving open-pollinated (OP) seed, as well as traditional control-pollinated breeding, where the complete pedigree is recorded and tracked. Polycross breeding can be used to maintain incomplete pedigree information, while achieving reliable estimates of breeding values for female parents. For polycross and OP systems, molecular marker methods can be used to reconstruct the unknown parts of the pedigree and thus improve estimates of breeding value and manage accumulation of relatedness.

The progeny in recruitment populations must be assessed in some way to infer information about their genetic values and those of their relatives. This can be as simple as visual assessment of phenotype in family blocks, but can be improved by employing stronger statistical tools such as Best Linear Unbiased Prediction (BLUP) and experimental designs to improve the accuracy of estimated breeding values. Genetic marker information can also be incorporated into the BLUP (marker-assisted BLUP) or associated at a genome level with phenotypic performance (genome-wide selection).

After evaluating the basis for a breeding programme in terms of programme objectives and the options available considering biological, economic, institutional and soci-political factors, there will usually be a variety of possible strategies and tactics that could be used to reach programme objectives. There is seldom a simple "best" choice.

As an illustration, we consider alternatives for breeding of Scots pine (*Pinus sylvestris L.)* in central Sweden. Starting with 50 progeny-tested founders representing a typical stratum in a multiple-population breeding structure, computer simulation was used to estimate the changes in genetic

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parameters that would occur under six mating and selection scenarios, described and evaluated in terms of time and resources used, potential genetic gain achieved and genetic diversity maintained. The alternatives represented both backward and forward selection, "expansion" of the BP, polymix and controlled pair mating, and clonal replication of field tests. While the breeding cycle lengths varied markedly among scenarios, dimensions were adjusted so that scenarios were compared at equal levels of effort and equal loss of proportional gene diversity per year, for three levels of investment.

In general, strategies using progeny testing for backward selection are very accurate; producing the highest gain per cycle, but the long cycle times reduce annual gain as compared to strategies using forward selection. The simplest forward-selection programme selecting the final BP directly in field experiments with control-pollinated trees results in greater annual gain. The annual gain can be further improved by using clonal replicates to improve selection accuracy, despite decreased selection intensity when using the same annual resource and the extra time required for cloning.

The gain by forward selection can also be improved by "expanding" the BP, from 50 to 100 in our example. The trees of an expanded BP can also be progeny tested using a pollen mix of the selected BP-members. By selecting an excess of candidate tress for paternity analysis, using DNA markers, a new BP can be selected forward in the polycross families, while maintaining a genetic diversity target; the higher the target, the more paternity determinations are needed. In general, strategies using forward selection also produce relatively higher annual genetic gain at lower resources compared to those using backward selection, since there is a starting cost in terms of trees used for progeny testing or ramets used for clonal testing.

Our illustration shows a change in rank order among strategies, depending on how the comparisons are made. It emphasises the importance of adjusting parameters appropriately to make a fair comparison. Our results are based on computer simulation, which is of great help, but not all decision factors can be incorporated in a simulation model. In a real situation, each strategy must be optimised and the sensitivity of result comparisons analysed. This might include the influence of model parameters like heritability, but also changes in technology that might improve selection accuracy, crossing and mass propagation options, and their effects on breeding-cycle time. Finally, we emphasise that the genetic gain in progeny of a selected subset deployed to a seed orchard or clone mix should be used to evaluate a breeding strategy, rather than the mean of the breading population.

The research leading to these results has received funding from the European Community's Seventh Framework Programme (FP7/ 2007-2013) under the grant agreement n° 211868 (Project Noveltree)

METAGENE, a simulation tool based on finite loci approaches: new developments to consider phenotypic plasticity in forest tree breeding.

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METAGENE is a simulation tool based on a finite loci approach for populations under artificial selection. This tool has been mostly developed within NOVELTREE project to address specific issues concerning the optimization of breeding strategies. In a first brief part of the presentation, we will introduce some of the key features of this tool and point at some of the examples in which the tool has been used. Tools like METAGENE are routinely used to predict the dynamics of quantitative traits in selected populations, where stochasticity is used in the sampling of alleles at each locus, mimicking recurrent recombination and segregation processes between loci. By using explicit models of gene action, METAGENE can conveniently address the question on how the underlying genetic architecture of selected traits affects their genetic improvement, for instance when co-selecting correlated traits or when considering non-additive causal components. This feature of models of explicit gene action has been used for simulating MAS schemes in NOVELTREE.
In the second and more detailed part of the presentation, we will introduce some new features of METAGENE that have been developed recently in the framework of NOVELTREE. These features take the explicitness of gene action one step further, notably by adding the possibility of gene-to-gene and gene-to-environment interactions as causal components of quantitative variation. The aim here is to go beyond the "additive paradigm" that characterizes most quantitative genetic models used in simulations. Indeed, evidence is accumulating that epistasis is a pervasive phenomenon, which hints at the complex genetic networking that causes genotype-by-environment interactions and plastic responses. By extending METAGENE features in this context, we wanted to model quantitative traits that respond to environmental cues in a plastic way. Plasticity traits or traits described by norms of reaction are receiving an increasing interest in plant biology. Plasticity for certain traits could have an adaptive value, notably for plants and trees, and be the escape to rapid and unpredictable environmental changes. METAGENE models plastic phenotypic responses by two genetic mechanisms: epistatic regulators that respond to environmental clines and modulate the effect of other genes in a gene network, and genes that change directly their effects over an environmental gradient, known in this context as allele sensibility or pleiotropy.
We illustrate the use of these mechanisms with an example in which a plasticity trait is studied over different experimental setups, comprising alternatively clones, full-sib families or half-sib families. Norms of reaction are constructed either between clones over environments or between (half or full) sibs over the same environmental gradient. The aim is to ascertain to what extent the use of families for assessing phenotypic plasticity produce biased results with respect to clonal variation over environments.

Keywords:
Finite allele model, simulation, epistasis, pleiotropy, phenotypic plasticity.

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Prediction of breeding values maximizing data from trials over 76 sites.

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The analysis was based on a large series of multiple environment tests in which 2493 parents where tested in trials comprising 317,000 trees over 76 trial sites with varying degrees of parental connectivity between sites. The analysis was confined to 6 traits of primary interest to the marketing of radiata pine seed in New Zealand and Australia. These 6 traits were diameter at breast height, straightness (score 1-9), branch cluster frequency (score 1-9), Dothistroma resistance (percentage of damaged foliage), wood density and stiffness based on sonic velocity assessed on standing trees. Diameter, straightness and branching were assessed on each tree. Dothistroma resistance, wood density and stiffness were assessed on a smaller sub-sample of trees. The objective of the analysis was to produce accurate breeding values and where possible indicate subgroups of sites on which particular estimates were more appropriate.

The approach to the analysis followed the models described in Beck *et al*. (2010) and Cullis *et al.* (2010). Given the size of the data-set and the number of sites and trees we used the so-called reduced animal model to model the additive genetic by environment effects. Preliminary work suggested that there was little loss in accuracy using this approximation. The key components of the analysis included

- Examination of parental site-connectedness. Sites with poor connectivity were excluded from the analysis
- MET analysis using a sequence of factor analytic models for the additive genetic by environment effects
- Clustering of the environments based on the best fitting factor analytic model as described by Cullis *et al.* (2010)
- Prediction of overall and cluster breeding values

The amount of additive genetic by environment interaction (AxE) for each trait was computed as a summary measure and this indicated that diameter had the most AxE with a value of 70 percent with the other traits having smaller values between 10 and 29 percent. The order of the factor analytic model varied from 1 to 3. For diameter the fa3 model explained 87 percent of the variance. In all traits the fitted factor model explained more than 80 percent of the total variance.

The sites clustered into different groups depending on traits. Diameter clustered into two major groups with other trials being isolated into groups of one or two trials. Those trials in isolated group were identified in terms of unusual environments or particular issues in terms of trial establishment and early growth. Clusters for the other traits indicated either differences in assessment technique or poor sample on a cluster of sites.

There is a large amount of noise in terms of variability in trial establishment and the early growth years and the climate data is based on relatively large polygons. Developing a predictive relationship between climate and the clusters is still a work in progress. It is clear that trial design and establishment need to be combined to reduce noise. More accurate climate data potentially using portable weather stations will provide better data to relate the cluster based on assessment data to site effects. The methodology of using the reduced tree model and factor model over sites has been very effective and breeding values are now available for clusters and overall sites. This analysis has provided breeding values which may be more useful in terms of creating environment-specific selection indices.

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A case study in marker-assisted breeding for disease resistance: the building of complex resistance to *Melampsora larici – populina* in hybrid poplar.

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The foliar rust caused by *Melampsora larici-populina* Kleb. (*Mlp*) is the main disease affecting poplar stands in northern Europe. Selection for durable resistance to this pathogen is an important challenge for poplar breeders. Past selection programs, usually using field phenotypic evaluation under severe natural infection, led to a one-by-one deployment of few qualitative resistance genes in highly related commercial clones. The high adaptive potential of the pathogen and the low diversity of poplar clonal stands enabled the overcoming of the deployed resistances. Quantitative broad spectrum resistances and pyramids of qualitative resistance genes (even defeated) are considered as potential ways to impair adaptation of the pathogen thus providing durable resistance. Markers are attractive tools to track resistance genes underlying these qualitative and quantitative resistances while avoiding an extremely labor intensive phenotypic screening involving several strains of known virulence. This is particularly useful to follow minor resistance genes of which the expression can be masked by epistatic effects of major resistance genes when challenged with *Mlp* strains or populations that do not overcome these major resistance genes.

In order to compare phenotypic selection and marker-assisted selection in the building of a complex resistance to *Mlp*, we used quantitative rust resistance phenotypic data and an updated genetic map from a *Populus deltoides* X *P. trichocarpa* F_1 progeny. The progeny was assessed for three quantitative resistance components measured in the laboratory using seven *Mlp* strains (Dowkiw *et al*., 2003; Dowkiw and Bastien, 2004; Jorge *et al*., 2005), a field susceptibility component measured in three sites and tolerance components measured as differences in growth (height, girth or volume) in an untreated *vs.* fungicide-treated field trial. A library of 7.5 million high-quality SNPs was identified and extracted from 19 resequenced genomes (Slavov *et al*. 2012; Geraldes *et al*. 2012). Selected SNPs were used to develop a 6K *Populus* Infinium SNP array with capability to assay 5,390 unique SNP loci. Genotyping data were used to generate 750 markers whose segregation did not differ significantly ($P(c^2) < 1‰$) from the expected 1:1 ratio. A framework genetic map was built with these genotypic data together with previously obtained data (including 800 loci, RAPD, RFLP, AFLP and SSR markers; Jorge *et al*., 2005; Bresson *et al*., 2011). Parental genetic maps were constructed with Carthagène (Givry *et al*., 2005) using the pseudo-testcross strategy (Grattapaglia and Sederoff, 1994).

A linkage map with 38 linkage groups was obtained (19 for each parent). The total length of the *P. trichocarpa* map was 5320 cM, with a mean distance between successive markers of 8.9 cM. The total length of the *P. deltoides* map was 4028 cM, with a mean distance between successive markers of 13.3 cM. A sum of 705 and 337 framework markers was included in the *P. trichocarpa* and *P. deltoides* maps, respectively. A QTL analysis was performed to estimate position, confidence intervals and additive effects of all detectable loci associated to broad and specific quantitative resistance and field tolerance. Phenotypic selection was compared to different MAS scenarios where influence of parameters such as family size, marker density, allelic effect, on selection results was investigated through simulations. Both phenotypic selection and MAS selection was empirically applied to the available progeny and relative efficiency of MAS in terms of gain in time, gain in experimental costs and management of disease resistance genes were determined.

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Eucalyptus globulus vegetative propagation in gPS.

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The PortucelSoporcel group (gPS, www.portucelsoporcel) is dedicated to produce and market high quality paper for office and offset uses. Portucel is Europe's largest producer of bleached eucalyptus kraft pulp. It is also one of the five largest European producers of uncoated wood-free paper. The company traditionally uses *Eucalyptus globulus* as the prime raw material for the production of pulp and fine printing and writing paper.

RAIZ (http://www.raiz-iifp.pt/) forestry & paper research institute belongs to the gPS group, and is committed to support the competitiveness of the Portuguese Pulp and Paper Industry, through research, technology transfer and training. Its mission results from the applied research needs of the industry, and recurring efforts to optimize the cost/benefit ratio of forestry and industrial practices, in order to assure its sustainability.

Eucalyptus globulus is the main species planted in Portugal, and is a benchmark in pulp & paper production, due to its high basic density, fiber morphology, lignin content/quality and pulp yield. Cloning elite material allows the maximization of genetic gains obtained through breeding (i.e. improved productivity, tolerance, adaptation and/or wood properties). The adventitious rooting ability of the selected clones is crucial in the deployment stage. Vegetative propagation is still a challenge in *E. globulus*. Many elite genotypes are discarded from the genetic improvement program, due to adventitious rooting limitations. There is variation in this trait between clones, but also within the same clone. The architecture of the rooting system also varies, influencing the performance of clonal plantations in Portugal. We will refer to ongoing studies on the nutrition, substrates, rejuvenation strategies, pest control and cuttings chemistry.

Breeding and deployment guidelines for durable resistance to *Melampsora larici-populina* leaf-rust in interspecific hybrid poplars.

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During the last century, several interspecific hybrid poplar cultivars with qualitative (i.e. complete) resistance to the Eurasian foliar rust pathogen *Melampsora larici-populina* (*i.e. Mlp*) were released in Europe, but the pathogen overcame the newly deployed resistances one after the other (Steenackers *et al*., 1996). The situation became critical when *Populus trichocarpa x P. deltoides* cv. 'Beaupre´ became susceptible as a result of the emergence of virulence factor 7. As this cultivar was covering large acreages in Belgium and France, a severe epidemic of the pathogen ensued.

Several major outputs from the NOVELTREE project contribute to a general rethinking of the breeding and deployment strategies to achieve durable control of the pathogen.
Unprecedented efforts were directed at the study of quantitative resistance (QR), especially in the co-evolved parental species *P. nigra*. We demonstrated the existence of a large reservoir of variation for QR in the species, with some evidences of geographical pattern. Although significant additive effects were detected, non-additive effects predominate thus making genetic prediction more difficult and individual selection more laborious. Strain-specificity being the rule rather than the exception, the relative benefits of using mono-strain experiments as opposed to mixed-inocula or field experiments appears more clearly now. Although responsible for the present situation, the qualitative resistances inherited from the exotic species *P. deltoides* were reconsidered under three aspects: (i) what is the breeding value against *Mlp* of this parental species when qualitative resistance is absent or defeated? (ii) is R-gene pyramiding a valuable strategy? and (iii) can we identify durable sources of qualitative and quantitative resistance ? The NOVELTREE project also allowed considerable progress in the study of traits that are distinct from resistance and still alter the effects of the pathogen. Rust avoidance and rust tolerance as measured in fungicide-treated *vs* untreated field experiments both exhibited significant genetic variation in *P. deltoides* x *P. nigra* hybrids. The genetic correlation observed between rust resistance and rust tolerance was of moderate level which let breeders expect higher genetic gain through multi-trait selection.
Characterizing the composition of the pathogen's populations in terms of virulences and pathotypes over the project's time frame in multi-site field experiments shed new light on the spatial and temporal aspects of resistance breakdown. Selection for virulence is both rapid and local, while unnecessary virulences do not seem to be purged out. The origins of pathogen adaptation to a newly released resistance factor also appears more clearly now. Tracking strains of *Mlp* able to circumvent a major QR factor inherited from *P. trichocarpa* in both naïve and trained pathogen populations revealed significant pre-adaptation in the pathogen. Finally, erosion of quantitative resistance following recurrent selection for improved aggressiveness was evaluated. Performances of genotypes in both monoclonal and clonal mixture plots confirmed the importance of the high initial selection pressure exerted by the host which favour local proliferation of the most adapted *Mlp* individuals in an epidemic process which could involve more than 12 cycles per year. Consequences for optimal temporal and spatial deployment of new clonal cultivars are finally discussed.

The research leading to these results has received funding from the European Community's Seventh Framework Programme (FP7/ 2007-2013) under the grant agreement n° 211868 (Project Noveltree)

Posters

Improving Sitka spruce in Britain: first steps towards a molecular approach.

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Background

Although native to the Pacific North West of Canada and USA, Sitka spruce (*Picea sitchensis* Bong. Carr.) is well suited to the moist, warm climate of northern and western Britain where it has become the most important plantation conifer species, with approximately 700,000 ha now established. The UK Sitka spruce breeding programme, on-going for over half a century, is now in the second generation of selection. The breeding objective is to produce faster growing, straighter trees with fine branches and no associated decrease in timber strength. The programme has provided the UK forest industry with improved material in terms of these economically important traits without deterioration in construction grade strength requirements. Improved Sitka spruce planting stock has been widely adopted in Britain, with nearly 26 million (80 per cent) of the 32 million Sitka spruce trees planted in 2007 being from improved sources.

It is important that future plantations of improved Sitka spruce trees yield both a greater volume of sawlogs per unit area compared with current planting stock as well as an improvement in timber quality. Recent advances in assessing the phenotype in terms of timber quality using acoustic tools have made this a more achievable objective. There is now an appreciation that microfibril angle (MFA) and stiffness measured acoustically offer a better indication of timber quality than the cruder 'Pilodyn' measurement of wood density.

Recurrent selection for superior individuals within the best full-sibling families remains the principal means of advancing the current breeding programme, but this is by its nature a slow and expensive process.

Objectives

Recognising the potential of novel DNA technologies for the early selection of superior genotypes with the traits of interest, Forest Research established a large Sitka spruce clonal trial in 2005. This comprised of three full-sibling families, represented by 1500 offspring per family, each clonally replicated four times per site, and planted at three climatically contrasting locations in the UK. Unimproved control material was also planted as a standard at each site.

The ultimate objective of these large clonal field trials is to provide material that will underpin the development of novel molecular breeding methods that will allow accurate early selection of individuals with superior wood quality traits such as MFA and wood density. However, as the trial is not yet old enough to permit accurate assessments of these wood quality traits, height and the phenology of bud flushing were chosen to act as examples of proof of principle traits that could be used to explore the correlation between phenotypic field performance and molecular markers.

The aim of this study is to create a genetic linkage map for Sitka spruce using microsatellite and single nucleotide polymorphism (SNP) markers and subsequently to locate QTLs for height and bud burst on the map, enabling an understanding of the basic genetic architecture of these traits. Once the Sitka map has been created it will enable the level of synteny and colinearity with other *Picea* species to be compared and any potential QTL hotspots to be cross validated.

Methods

DNA was extracted from the six parents of the three full-sib crosses and from 306 offspring of a single cross. This included 50 individuals each of the consistently earliest and latest flushers, as scored across the three sites.
The SNP genotyping was carried out in collaboration with the Arborea group in Quebec. DNA was extracted with a Qiagen DNeasy kit to a minimum concentration of 50ng/µl and shipped on dry ice to Quebec, where it was applied to an Illumina iSelect 16,720 bead array developed for gene-coding SNPs in white spruce. This chip was designed as a one SNP per gene assay in order to map the greatest number of genes onto the genome of a single pedigree population. The microsatellite loci used in the construction of the Sitka map comprised both neutral and EST based markers.
The trial was phenotyped for all replicates of one full-sib family at all three sites. Bud flush was scored by visual inspection using a delineated 8 stage flushing scale and height was assessed as the total height (m) measured at the end of the growing season following terminal bud set.
Marker grouping and linked loci ordering were performed using a minimum LOD threshold value of 4.0 and a minimum recombination fraction of 0.4. All linkage analyses and map estimations were performed with Joinmap 3.0 using the mating type CP (cross-pollination).

Results
In total, 306 progeny were included in the map of which 102 had been genotyped at the microsatellite loci, 113 at the SNP loci and 91 at both SNP and microsatellite loci.
Two individual (parental) and one composite linkage maps were assembled from the 306 progeny. The iSelect array yielded a total of 14,145 SNP's of which 1,081 were informative and 961 were mappable in our Sitka cross (after parental allele identification issues). The map also included 115 informative microsatellite loci. For the female map, 655 SNP loci were informative. A total of 587 loci grouped onto 12 linkage groups, with 57 failing the chi-square locus genotype frequency test (expected Mendelian ratios, $p<0.01$)) and 11 loci remaining ungrouped. In the construction of the male map, 698 SNP loci were informative. 587 loci were grouped onto 12 linkage groups with 100 failing the chi-square locus genotype frequency test and 11 loci remaining ungrouped.
Linkage groups were generally stable for a LOD of 4.0 with a LOD value of 5.0 sometimes applied to obtain comparable groups from one parent to the other.
The final composite map had a length of 2,090cM using Kosambi's mapping function.
Associations between segregating genetic markers and the phenotypic variability of each trait will be determined for each trial site and for each individual parental map. QTL model analysis will be carried out using MapQTL 6.0 software.

Indirect genetic effects in trees change the heritable variance available for selection and our perception of their genetic architecture.

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Background

Heritable variation is essential for adaptive evolution in nature and for the genetic improvement of breeding populations in plants and animals (Falconer and MacKay 1996). Although classical quantitative genetic models have made a major contribution to the understanding of inheritance and response to selection, they have neglected the fact that individuals may interact. Such social interactions among individuals are a fundamental property of life, occurring in virtually all taxa (Frank 2007). With social interactions, the influence of an individual's genes may extend beyond the individual itself to affect other individuals with which it interacts. These effects are known as indirect genetic effects (IGEs), and occur when the genotype of an individual influences the phenotype of another (Griffing 1977; Muir 2005; Bijma 2011). Empirical studies on IGEs are sparse, and there is little understanding of the magnitude of IGEs and the correlations between direct and indirect genetic effects, particularly in plants.

In a first study, we aimed to investigate the incorporation of IGEs into a quantitative genetic model to account for competition, with a particular focus on forest genetic trials. In this context, we have used simulated data to evaluate the implications of modelling competition at the genetic level on the estimation of variance components and prediction of response to selection, considering different levels of relatedness and survival within a neighbourhood of a focal individual. In a subsequent study, we have investigated the genetic control of individual interactions in a large pedigreed field trial of *Eucalyptus globulus* to test whether interactions among trees are genetically based and, if so, how these interactions change our perspective on total heritable variation and impact response to selection.

Methods

We have used a variance partitioning approach to estimate the contributions of the direct and indirect sources of genetic variance influencing a trait, and implemented within the framework of linear mixed model methodology. In this sense, an individual's total breeding value (*TBV*) is partitioned into component for a direct breeding value on its own phenotype and a component for an indirect breeding value on the phenotype of its neighbours. Thus, *TBV* defines the total impact of an individual's genes on trait values in a population, and it is the variance associated with these total heritable effects (*Var*(*TBV*)), rather than the additive genetic component of phenotypic variance, that determines the potential of a population to respond to selection (Bijma 2011).

Results

Our simulation results indicated that estimates of (co)variance components and predictions of responses to selection may be inaccurate when IGEs arising from competitive interactions among individuals are not accounted for by statistical models used in genetic evaluation (Costa e Silva

and Kerr 2012). Our simulation results also indicated that the ability to detect low to moderate magnitudes of genetic competition effects was reduced as levels of genetic relatedness among interacting individuals decreased, with the reduction being magnified with lower survival. Genetic relatedness within a neighbourhood prevented the negative response to positive selection under the presence of genetic competition, and thus increased the utilization of the total heritable variance.

Analyses of field data for growth and disease infection traits in a *Eucalyptus globulus* trial have resulted in contrasting impacts of IGEs on total heritable variance (Costa e Silva *et al.* 2012). A negative covariance between direct and indirect additive genetic effects was observed for the trait affected by resource competition (i.e. growth). This is likely to be due to genetically competitive trees decreasing the resources available to their neighbours, and led to a reduction in the total amount of heritable variance relative to the variance estimated from the direct additive effects alone. In contrast, a positive covariance between direct and indirect additive genetic effects was observed for disease infection. This is likely to be due to genetically susceptible trees acting as a source of inoculum and increasing the exposure of neighbours to disease, which led to an increase in the total heritable variance relative to the direct genetic variance. Thus, our field trial results showed that IGEs arising from tree interactions increased heritable variation in disease infection to levels exceeding ordinary (direct) heritability, whereas they substantially reduced heritable variation in growth. Consequently, population response to selection in disease infection may exceed conventional expectations, whereas response in growth may be prevented despite considerable ordinary heritability.

Conclusions

Our results challenge the traditional perspective on the genetic architecture of traits measured in forest tree species, and indicate that knowledge of IGEs is essential for understanding response to selection. Thus, IGEs will need to be taken into account in genetic evaluation to optimize selection programs for genetic improvement of tree species. The IGEs appear to be driven by neighbour competition and infection, respectively. These processes have contrasting effects on the total heritable variance available for selection. If variances in IGEs and their covariance with direct genetic effects persist in natural populations, then neighbour competition is likely to constrain evolutionary progress in resource-limited traits, whereas neighbour infection is likely to amplify the evolutionary response over that predicted from considering direct genetic effects alone.

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Heterozygosity-Fitness correlations in Maritime pine (*Pinus pinaster* Ait.).

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Correlations between molecular-marker heterozygosity and fitness traits, also known as heterozygosity-fitness correlations (HFCs), have been examined for more than three decades in numerous organisms. These studies, in general, indicate a positive relationship between heterozygosity and fitness traits. Nowadays, the use of a high number of SNP marker data in the analysis of HFCs may help us disentangle the direct and indirect effects of polymorphisms, shedding light on this classic issue. Provided that most economically important forest trees, and particularly conifers, are outcrossing wind-pollinated species, often suffering strong inbreeding depression, a positive relationship between molecular-marker heterozygosity and fitness is also expected in these species. A maritime pine (*Pinus pinaster* Ait.) common garden experiment was established at four sites, covering distinct environmental conditions (from Mediterranean to Atlantic). Each trial comprised 650 clones from 37 populations (20,800 trees were phenotyped). At each clonal trial, we studied the correlation between observed heterozygosity at 12 nuSSRs and 266 candidate gene SNPs, and survival and total height, as fitness proxies. Variance components and clone BLUPs for both variables were estimated by restricted maximum likelihood (REML), using a linear mixed model approach for height, and a generalized linear mixed model for survival. Clonal heritability ranged between 0.27±0.03 and 0.47±0.07 for height, and between 0.04±0.01 and 0.16±0.08 (logit scale) for survival, at different locations. Observed heterozygosity ranged from 0.174 to 0.335, depending on population. Correlations between SNP heterozygosity and clone BLUPs for survival or total height were non-significant at the four sites. HFCs were neither significant when clones were pooled in metapopulations according to their genetic structure based on the 266 genotyped SNPs. Possible causes of our results are discussed based on the estimate both of the inbreeding load and the amount of variance in fitness explained by inbreeding.

Use of a relationship matrix based on markers to evaluate genetic values: a case study in maritime pine.

Bouffier L., Ruby M,. Chancerel E., Plomion C.

In traditional forest tree breeding programs, breeding values estimation requires a matrix describing the additive relationship between individuals which is currently derived from pedigree information. This approach supposed an unrelated base population (G0 population) and a complete knowledge of genotypes pedigree from the G0 population. An alternative to this average relationship matrix estimated from pedigree is to use marker information to infer relationships. To build this realized relationship matrix, no assumption is needed about G0 population relatedness and it allows to deal with incomplete pedigree due to open- or polymix-crosses. If the markers are sufficiently dense, such an approach could potentially be more accurate than using pedigree information (Hayes et al., 2009).
Maritime pine (*Pinus pinaster* Ait.) is the main plantation species in France. A breeding program has been implemented since the early 1960s using a recurrent selection scheme. The genetic evaluation is currently realized based on pedigree information. A preliminary study is here carried out to evaluate the interest of using molecular markers for the relationship matrix and evaluate the consequences on BLUP evaluation.
In that perspective, a maritime pine polymix progeny trial has been studied. This trial was measured for various traits relative to growth (diameter, height), wood quality (stem straightness, spiral grain, wood density) and adaptation (water use efficiency). The 50 parent trees were genotyped for 2500 SNPs displaying polymorphism in that population.
Genetic evaluation of these 50 parent genotypes will be carried out with an individual model where the relationship matrix will be built in three different ways:

- with pedigree information
- with SNPs information
- with both pedigree and SNPs information.

Comparison of breeding values estimated from these three different models should allow evaluating the interest of using the molecular data for the realized relationship matrix construction.

The research leading to these results has received funding from the European Community's Seventh Framework Programme (FP7/ 2007-2013) under the grant agreement n° 211868 (Project Noveltree)

Session 4: Perspectives for Forest tree Breeding for the next 20 years

Invited Talk

Accelerating the domestication of forest species using genome-wide selection and next-generation sequencing

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A rapidly changing and increasingly variable climate will require that tree breeders accelerate the improvement of germplasm better adapted to existing and new sources of biotic and abiotic stresses. In parallel, the demand for wood-derived products, particularly for bioenergy, is expanding at a rate that outpaces the gains in productivity. The challenge of rapidly developing better adapted and improved germplasm is enormous for tree species, where breeding is complex, expensive, and time-consuming. This limitation is a threat to the survival of natural and planted forests, with devastating consequences to the global carbon balance and global warming. To respond to these threats we are now hyper-accelerating conifer breeding in the U.S. to rapidly develop new, improved cultivars that are adapted to broad climate variation, resistant to existing and emerging pathogens, and increasingly productive. We recently demonstrated that early selection can be achieved using genomic predictive models developed for a breeding population of pine, that have higher accuracy than traditional selections based on phenotype alone. Models were developed for 17 traits, encompassing productivity, disease resistance and developmental properties. Furthermore, we now showed that newly developed models that incorporate dominance effect exceed the accuracies of standard (additive) models by more than 30%. Development of accurate models based on additive and dominance effects create the foundation for breeding by design –the concept that optimal mate-pairs can be identified *in silico* based on the prediction of the phenotype of the progeny based on the parental genetic composition. We have now applied this concept to identify, among over half a million possible crosses, the families that are predicted to be widely adaptable to climate variation, while being highly productive and disease resistant. Application of these strategies is increasingly affordable as novel genotyping strategies based on next generation sequencing are developed.

Oral presentations

Use of RAD sequencing for constructing linkage maps and genomic evaluation.

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RAD sequencing has been applied to a single full sib family of Sitka Spruce to generate tens of thousands of markers. The parents were sequenced to a high level of coverage so there was negligible ambiguity in the segregation of markers within each parent, but the 1500 offspring had only low coverage. The most informative markers from the two parents and 622 offspring trees were used to generate a linkage map, which resulted in 12 linkage groups containing 8194 markers with a total map length of 3511 cM. Following this a Genome Wide Association Study was carried out which identified a number of significant loci for height. Work that is being conducted before the meeting intends to extend this analysis to include bud burst and also to examine the accuracy of genomic evaluation.

The research leading to these results has received funding from the European Community's Seventh Framework Programme (FP7/ 2007-2013) under the grant agreement n° 211868 (Project Noveltree)

Genomic selection in small populations with reduced effective size: example of oil palm.

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Abstract
Genomic selection is expected to increase genetic gain per unit time in oil palm (*Elaeis guineensis*) but currently only simulation results exist (Wong and Bernardo 2008). We applied cross-validation on real data of progeny-tested individuals to estimate the accuracy of genomic selection in oil palm. This study used the largest dataset of estimated breeding values reported in oil palm breeding. It included two breeding populations of 100 and 130 individuals, genotyped with 200 SSR and phenotyped for five traits with a wide range of heritability. Deregressed estimated breeding values were used as observations in a weighted analysis to derive genomic estimated breeding values (Garrick *et al.* 2009). Two strategies were used for sampling training populations: within population structure based on K-means clustering (Saatchi *et al.* 2011) and across population structure. Several statistical methods were compared. The accuracy of genomic selection was estimated in the test population. Our results will be valuable for all breeding programs where populations are small and have a very small effective size.

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Marker-assisted breeding in *Pinus radiata*: From Candidate Gene-based selection to Genomic Selection

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New Zealand has approximately 1.8 million hectares of commercial plantation forests, of which 89% is radiata pine (*Pinus radiata* D. Don). Radiata pine contributes approximately 2.5% to New Zealand GDP and has been subjected to intensive breeding and propagation for over 60 years. We describe two different research projects underpinning application of DNA markers in selective breeding programmes used by the NZ forestry industry.

Our candidate gene-based association genetics (AG) programme was implemented to detect actual causal genes and sequence variants to inform both genetic modification and gene-assisted selection (GAS) research. Widespread utilisation of GAS was anticipated due to the low cost of implementation, particularly when used in combination with an array of possible propagation technologies to amplify superior radiata pine genotypes. However, various bottlenecks have restricted the number of causal polymorphisms detectable to such an extent that insufficient trait variation could be explained to warrant application. These bottlenecks included (1) the availability of suitably large association populations with which to detect sufficient associations between markers and underpinning quantitative trait loci with adequate statistical evidence, and (2) the cost associated with extensive candidate gene screening and resequencing, as well as genotyping and phenotyping large association populations, especially for multiple traits.

Recent technological advances in Next Generation Sequencing (NGS) have enabled the identification of many thousands of single nucleotide polymorphisms (SNPs) at a greatly reduced cost and timeframe. This has raised the possibility of genomic selection (GS), which utilises hundreds or even thousands of SNPs to predict the breeding values of genotype for traits of interest in multiple closed breeding populations. Moreover, research by colleagues working with other species has shown GS is now a viable option for large genome species such as radiata pine.

We have therefore commenced work in four areas collectively aimed at implementing GS by radiata pine breeders. Firstly, development of a SNP Chip will utilise the SNPs generated in our original GAS polymorphism discovery programme. In addition to public domain polymorphisms, SNPs will be data mined from NGS of multiple transcriptomes from a range of genotypes. To date, Illumina-based transcriptome sequencing of a single genotype yielded 164,145 SNPs with varying levels of *in silico* reliability, as determined by the number of different detection algorithms capturing a given SNP. In parallel, generation of a genome with 20X coverage within the next two years will further increase the SNP pool whilst providing a physical framework, integrated with existing genetic maps, in which to place polymorphisms. We are also addressing the lack of cost-effective DNA extraction protocols capable of meeting the quality requirements of high-throughput genotyping platforms that is currently a barrier to industry implementation. Finally, scenario testing for implementation of GS will best determine how to integrate GS into the existing breeding strategies once sufficient marker resources are available.

Forest ecogenomics and genomic selection for mitigating climate change and breeding resilient trees.

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Forest population and landscape genomics, molecular ecology and ecogenomics, and other genomics approaches, tools and resources provide researches now with unprecedentedly new opportunities to study plant genetic adaptation to environmental stresses at the genome-wide level and to do genomic selection for frost tolerance, drought resistance, and other important adaptive traits using simultaneously thousands of genes and millions of genetic markers that represent the entire genome variation. Global climate change, accelerating human population growth rate and, respectively, the growing demand for construction and energy tree products require a more efficient tree breeding. Conservation of forest tree resources has also become a very serious problem. The growing demand for an alternative, available, renewable, and ecologically friendly source of energy significantly increases the role of forests and population and ecological genomic studies of their adaptive, ecological, and economic potential. The accelerating global climate change can exceed the adaptive potential of pine forests and lead to their contraction on large areas. However, some forest tree populations growing now in the regions that have ecological conditions similar to the future changes are likely to possess the adaptive potential necessary for spreading adaptation into other regions with similar ecological conditions expected in the future. Unfortunately, phenotypic plasticity and the historically established gene flow between remote populations can be insufficient for fast natural propagation of genetic adaptations. In this connection, the role of conservation management and especially that of assisted migration (also called assisted colonization, managed relocation or translocation done by physically moving the plant material (pollen, seeds and seedlings) to other regions where this material is better adapted to the future environments) increases. Modern methods and tools of population, landscape, and ecological genomics allow to study the genetic basis of adaptation in forest tree species and also detect the main genes responsible for important adaptive and economic traits that can be used in assisted gene migration and tree breeding for biomass growth, water use efficiency, cellulose content, and other traits important for adaptation and for bioenergy and biofuel production. The recent data on Douglas-fir (*Pseudotsuga menziesii* var. *menziesii*) and loblolly pine (*Pinus taeda* L.) populations studied for thousands different genes are presented to illustrate population and ecological genomic studies in conifers.

Trees4Future, a European Research Infrastructure project.

Fluch S., Pâques L.

Trees4Future, a European Research Infrastructure project, aims to integrate, develop and improve major forest genetics and forestry research infrastructures in the area of wood products and forest services. It will provide the wider European forestry research community with easy and comprehensive access to currently scattered sources of information expertise *via* a central portal as a gateway for accessing distributed data sources. Information for various user groups includes all types of forest information from research infrastructures, databases to research results in the context of genetic adaptation and changing climatic conditions. Standardized material and protocols will help to increase our knowledge about the adaptation of forests to climate change and tree characteristics suited for tailor-made wood supply - thus optimising the short- and long-term exploitation of forest resources.

Special focus of the project is the creation of a new and better linked research infrastructure landscape. The Transnational Access programme as a tool for promoting the infrastructure offers external users free access to 28 state-of-the-art forest research structures and facilities across Europe. The facilities focus on genetics, genomics and tree breeding, (eco-)physiology and biotechnology, wood science and technology and modelling/data analysis. They include genetic databanks, gene banks and DNA collections, various laboratories, models and decision-support systems.

Posters

Development and validation of a 12K SNP chip genotyping array for association genetics in *Populus nigra* using genome-wide resequencing data.

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Recent developments of high throughput SNP arrays allow accurate genotyping of hundred to several thousand loci for many individuals at relatively low cost. The ability to determine genotype at many loci for many individuals allows genetic studies that cover more genomic variation useful for population genomics, hybrid controls and monitoring of genetic diversity in natural and breeding populations. We took advantage of these new technologies to design a large SNP array for QTL mapping and association genetic studies for different traits of interest in *Populus nigra*. A major issue in association studies is the mapping approach to undertake: a whole genome scan or a candidate gene approach. In poplar, due to the relatively high SNP frequency (about 1 every 100 bp) and the low linkage disequilibrium (R^2 measures of LD fall to half their value within 300 bp to 1 kb) a whole-genome scan is not yet recommended. We have constructed an Illumina Infinium 12 K beadchip array.
Our global strategy was (i) to cover the entire genome with a minimum SNP density (1SNP/200kb), and (ii) to densify in SNP candidate regions on the basis of QTL hot spots and candidate regions for wood properties, bud phenology, rust resistance and drought resistance. The aim of this strategy is not only to optimize the chance to detect associations but also to be able to account accurately for genetic structure in the association studies.
The SNP resources used were retrieved from Sanger re-sequencing candidate genes and from whole genome re-sequencing of different *P. nigra* panels (21 to 51 individuals). Firstly, SNP discovered through Sanger re-sequencing of almost 700 candidate genes (full or fragments) involved in photoperiodic signaling pathway, or differentially expressed in relation to wood properties and rust resistance. Secondly, we took advantage of next-generation sequencing (NGS) technology to extent the identification of SNP at whole genome scale. In the frame of a collaborative project supported by the network of excellence EVOLTREE (http://www.evoltree.eu/) and the two FP7-EU projects NovelTree (http://www.noveltree.eu/) and EnergyPoplar (http://www.energypoplar.eu/), we undertook an hybrid approach combining deep coverage re-sequencing of 4 individuals (20X) and moderate (10 X) to low (2X) coverage re-sequencing of 47 individuals. Individuals were chosen to represent genetic diversity of the latitudinal range of *P. nigra* mainly in Western Europe. Libraries and paired-end sequencing (2x75bp and 2x100 bp) on GAIIx or HiSeq sequencers (Illumina) were performed by IGA (Applied Genomics Institute, Udine, Italy) and by EPGV group at IG-CNG (Institut de Génomique-Centre National de Génotypage, Evry, France). A home-made pipeline developed at IGA was adopted to extract the suitable SNPs for the Illumina beadchip using the whole set of polymorphisms detected on the *P. nigra* panel made of the 51 genotypes. A reference-guided assembly of the short reads of the French clone 71077-308 was run *versus* the *P. trichocarpa* genome sequence

v2.0, followed by the extraction of a *P. nigra consensus* sequence which was masked for duplications and repetitions. The reads coming from each deep re-sequenced genotype were aligned to the masked *P. nigra consensus* sequence in order to detect with stringent parameters a dataset of informative SNPs, which were eventually confirmed by the alignment of reads coming from the whole panel of low-coverage re-sequenced clones. The following parameters were then applied to select the SNPs: i. the coverage of the locus, ii. a minimum number of 5 genotypes must support a SNP, iii. the absence of SNPs and/or DIPs in the 60-bp flanking regions, iv. the loci within repetitive or duplicated regions of the genome were discarded. A total of 189 616 SNP were found on the genomic regions underlying QTL, candidate genes and control loci. A list of gene models selected on the basis of transcriptome studies and putative function based on literature was also considered to select SNPs outside candidate regions. SNPs referred as control loci were also selected outside QTL region and candidate genes.
The total collection of the SNPs (Sanger and NGS data) and the 60 bp flanking sequences were submitted to the ADT Illumina software to identify SNPs suitable for the design of the array, and 70 % of them showed the minimal required ADT score $\geq$ 0.6. Then, SNPs were preferentially selected in genomic area (within and outside gene space) showing QTL with large effect for phenology and rust resistance (80 SNP/ Mb). In genomic area displaying QTL with moderate effect or without target QTLs, SNPs were retained at lower density, 20 SNP/Mb and 5 SNP/Mb respectively (Jorge *et al.,* 2005, New Phytologist 167: 113-127; Rae *et al*, 2008, Tree Genetics and Genomes, 4, 97-112; Rae *et al*, 2009, BMC Plant Biology, 9:23; Rohde *et al*, 2010, New Phytologist 189: 106-121; Fabbrini *et al*, 2012, BMC Plant Biology 12: 47. DOI:10.1186/1471-2229-12-47). Among these SNPs only those having the following criteria were considered: i. Illumina score $\geq$ 0.85; ii. BLASTn hit against the *P. trichocarpa* genome sequence V2.0 with at least 97% identity and 120-nt length; iii. and a minimum of 24 informative accessions. A final number of 10 332 loci were included in the array corresponding to 12 000 beads requested by the Illumina Infinium chip assay.
In this presentation, we will also present preliminary genotyping data with a special focus on quality control and reliability of the chip. An association mapping population of 1000 individuals covering the latitudinal range of the western part of *P. nigra* native land will be genotyped. Diversity and structure description, and the association between genotypes and phenotypes will be performed. Data from this assay will yield important information about the feasibility of the current strategy in forest tree breeding.

Keywods:
P. nigra, large scale SNP discovery; association genetics, HT genotyping design

The research leading to these results has received funding from the European Community's Seventh Framework Programme (FP7/ 2007-2013) under the grant agreement n° 211868 (Project Noveltree)

High throughput phenotyping tools for analysis of poplar wood chemical composition.

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Selection and breeding of tree species requires high-throughput characterization of the wood properties in relation to both applications for material and bioenergy use. At the Laboratory of Wood Technology advanced techniques have been developed for this purpose. A considerable part of the activities was conducted in the framework of the European research project Noveltree co-ordinated by INRA. First, coupled DSC-TGA can be used as a tool to obtain semi-quantitative information on the chemical composition due to the different thermokinetics of the main constituents (lignin, cellulose and hemicelluloses) and gives also a detailed view on the energetical content of the material. Second a method is developed for using short-wave infrared signals to obtain chemical information on cellulose and lignin composition in a fast and quantitative way. Third, structural properties on different scales are acquired using the multi-resolution X-ray tomography set-up developed at UGCT, the Ghent University Centre for X-ray Tomography. The system offers a large range of operation freedom, all combined in versatile acquisition routines (standard or fast scanning, tiling, helix, etc.). Simultaneously with 3D structural information, (micro)densitometrical detailing is obtained as well. Combining all aforementioned techniques allows for quantitative characterisation of key wood properties for selection and breeding taking into account not only mean values, but surely also asses variability within and between trees.
The work mainly focussed on high throughput phenotyping tools to determine the macrochemical composition of poplar wood. Techniques should provide information not only on mean values but a major effort is related to assessing variability in wood constituents and their potential impact on wood properties related to both material and energy applications. A tiered approach was developed using wood discs for camera based analysis with short-wave infrared signals as proxy complemented with very small uniform samples linked to a selected point on the disc analysed through the DSC-TGA protocol. Next to this detailed mapping of macromolecular distribution also related wood structural characterisation is possible by means of linkages between tension wood determination based on the chemical analysis and helix X-ray tomography.

Keywods:
Poplar, high-throughput, chemical composition, DSC-TGA, SWIR, helix X-ray tomography

The research leading to these results has received funding from the European Community's Seventh Framework Programme under the grant agreement n° 211868 (Project Noveltree)

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Water use efficiency and drought responses in *Populus nigra* assessment from an automated irrigation control device.

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In forestry, water-use efficiency (WUE), the ratio of biomass produced to amount of water used, is a critical link between wood production and water management. WUE is closely related to the intrinsic water use efficiency (Wi). The effects of water deficits on plant productivity and WUE have been extensively reviewed, and measurement of WUE has been simplified by the discovery of a strong correlation between WUE and ^{13}C isotope composition. Isotope discrimination has been suggested as a sensitive long-term monitoring tool of physiological changes. Mainly assessed at leaf or wood levels, ^{13C} D has become a widely used indirect tool to investigate both genetic and environmental controls of Wi in crop species and forest trees. One important outcome of NovelTree was the development of a high throughput phenotyping technique to measure WUE in a large number of poplar tree genotypes. Possibilities of automation of irrigation control and assessments of growth and water-use related traits have been explored in order to offer new phenotypic tools to apply at large scale. Here, we present the results about the impact of controlled drought on transpiration efficiency (TE) at leaf level, whole plant level. Six *Populus nigra* genotypes were grown under two water regimes. We recorded: (i) ^{13}C content in leaf; (ii) ^{18}O enrichment in leaf; (iii) leaf-level gas exchange and (iv) biomass accumulation and water use by whole plants.

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Promoting a functional and comparative understanding of the conifer genome-implementing applied aspects for more productive and adapted forests

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In the midst of a climatic change scenario, the genetics of adaptive response in conifers becomes essential to ensure a sustainable management of genetic resources and effective breeding. Conifers are the target of major tree breeding efforts worldwide. Advances in molecular technologies such as, next-generation DNA sequencing, could have an enormous impact on the rate of progress and achievements made by tree breeding programmes. These new technologies might be used not only to improve our understanding of fundamental conifer biology, but also to address practical problems for the forest industry as well as problems related to the adaptation and management of conifer forests. In this context, ProCoGen will address genome sequencing of two keystone European conifer species. Genome re-sequencing approaches will be used to obtain two reference pine genomes. Comparative genomics and genetic diversity will be closely integrated and linked to targeted functional genomics investigations to identify genes and gene networks that efficiently help to develop or enhance applications related to forest productivity, forest stewardship in response to environmental change or conservation efforts. The development of high-throughput genotyping tools will produce an array of pre-breeding tools to be implemented in forest tree breeding programmes. ProCoGen will also develop comparative studies based on orthologous sequences, genes and markers, which will allow guiding re-sequencing initiatives and exploiting the research accumulated on each of the species under consideration to accelerate the use of genomic tools in diverse species. ProCoGen will integrate fragmented activities developed by European research groups involved in several ongoing international conifer genome initiatives and contribute to strengthening international collaboration with North American initiatives (US and Canada).