



Promoting Conifer Genomics Taking research a step further

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Picea abies



Picea sitchensis

Foreword

The first newsletter was an introduction to ProCoGen and reported the work progress of the first one and a half years. This newsletter presents the updates from 18 - 30 months of this project. The past months have witnessed several breakthroughs in conifer genomics. The genome sequences of *Picea abies*, *Picea glauca* and *Pinus taeda* which have been released recently are proving to be a great asset for the sequencing, assembly and genomic studies of related conifer species, such as those being investigated on this project namely *Pinus sylvestris* and *Pinus pinaster*. The close collaboration and integrating efforts of ProCoGen with other initiatives worldwide is contributing towards advancements in conifer genomics and in development of breeding tools for economically and ecologically significant tree species of Europe.

Overview

Genomic resources Sequencing of *Pinus sylvestris* and *Pinus pinaster* are in progress. The preliminary results have been used for comparative studies using data available from other conifer species. SNPs (single nucleotide polymorphisms) are being traced using probes from the unigene assembly of *Pinus taeda*. SNP database for *Pinus pinaster* has been set up and is augmenting the existing Sustainpine database established on the Sustain Pine project.

Adaptive capacity Protocol for transcriptomic analysis of conifers using laser capture micro-dissection and 454 pyrosequencing has been developed by Cañas et al. 2014 as a part of work package two. Preliminary transcriptomic analysis has shown differential gene expression in 14 different cells and tissues under study. Epigenetic studies on somatic embryos of *Pinus sylvestris* showed temperature dependent alteration of gene expression. ProCoGen also supported the epigenetic studies on *Pinus pinea* (Sáez-Laguna et al. 2014).

Comparative genomics A pilot exome capture has been designed to be tested on different pinaceae species. Broad scale comparison and microsynteny studies have been performed between *Picea abies* and *P. glauca* (De la Torre & Ingvarsson 2013).

Computational genomics Bioinformatic tools have aided the development of COS markers based on available sequence data from six different conifer species. A preliminary catalogue of SNPs from *Pinus pinaster* has also been compiled.

Translational genomics & breeding tools A proof of concept experiment is being developed for the implementation of genomic selection in maritime pine. The first preliminary study has given insight into genome wide diversity patterns and linkage disequilibrium in breeding populations (Plomion et al. 2014)

Dissemination and integration Apart from publications in peer reviewed international journals, ProCoGen partners have also been actively participating and disseminating the outcomes of this project at various national and international events. This project has also been offering interactive platforms and possibilities of transferring knowhow through dissemination and training workshops, staff exchange programme and via internet.

Work Package Objectives and Progress in Brief

Development of Genomic Resources (Work Package 1)

Objectives | To develop both *de novo* genome sequences and extensive catalogues of genetic variation for two species of pines: Scots pine (*Pinus sylvestris*) and Maritime pine (*Pinus pinaster*). The next generation sequencing has opened new doors for tackling conifer genomes, and the availability of sequence data from *Picea abies*, *Picea glauca* and *Pinus taeda* will be a great asset for assembling and resequencing related conifer species. In addition, SNP (single nucleotide polymorphism) discovery through resequencing is a promising tool in studying ecological genomics of local adaptation. This project will also greatly contribute to integrating European and Trans-Atlantic efforts, and will take conifer genomics a step further.

Main results from 18 to 30 months

- *Pinus sylvestris* sequencing strategy- Whole genome shotgun sequencing from a single megagametophyte sequencing (target coverage ~50x) from two paired-end libraries (150bp and 650 bp insert sizes) using Illumina technology as well as mate-pair libraries (5kb and 8kb insert sizes) from diploid tissue are being used.
- *P. sylvestris* genome sequencing- Whole Genome Sequencing (WGS) assemblies from shallow sequencing were produced using CLC Bio *de novo* assembler. Protein-coding and non-coding regions from the draft genome assembly have been compared with other conifer assemblies available for comparative evolutionary study in conifers. Comparative sequencing also revealed that the extant conifers shared the transposable element diversity. 454 reads from randomly sheared genomic DNA were used to identify and characterise transposable elements. LTR-RTs (long terminal repeat-retrotransposons) were identified as the most abundant in a comparative analysis with other conifer species. (Nystedt et al. 2013).
- *Pinus pinaster* sequencing strategy- 454 technology is being used for whole genome shotgun sequencing (target coverage ~1x) of DNA from a callus derived from a single megagametophyte (Arrillaga et al. 2014) using paired-end libraries (3kb, 6-8kb and 10-12 kb insert sizes).
- *P. pinaster* genome sequencing- 7 Gbp (giga base pairs) have been sequenced so far and further sequencing is underway.
- SNP discovery- *P. sylvestris* samples from six different populations (60 individuals,



Pinus pinaster



Pinus sylvestris



Pinus sylvestris



Pinus pinaster

one megagametophyte per individual) are being screened by exome sequencing using capture probes from *Pinus taeda* unigene assembly. (Neves et al. 2013, Neves et al. 2014). In case of *P. pinaster* SNP discovery is based on RNAseq re-sequencing technology in 96 diverse individuals. SNP database is available at: http://bioinformatics.psb.ugent.be/supplementary_data/procogen/ *P. pinaster* transcriptome characterization has been performed using illumine next generation sequencing platform. Polymorphisms could be identified from the sequencing data. The SNPs discovered in this study will further enhance the Sustainpine DB that has already been established (Canales et al. 2013).

Discovery of Adaptive Capacities (Work Package 2)

Objectives | To study the functional regulation of growth and adaptive responses to abiotic stresses via transcriptome sequencing, transcript expression profiling, small RNA (sRNA) discovery and profiling, identification of transcription regulatory networks and characterization of epigenetic dynamics in contrasting developmental and environmental conditions. The functional regulation of growth and adaptive responses will be focused on: drought stress studies in *Pinus pinaster* and cold acclimation in *Picea abies* and *Pinus sylvestris*. The molecular tools developed in earlier national and international projects will form the stepping stones for intensive studies in this project. ProCoGen will integrate the outcomes from other European initiatives like SUSTAINPINE, NovelTree, EVOLTREE and other European initiatives, creating a new dimension in conifer research.

Main results from 18 to 30 months

- cDNA libraries have been constructed from RNA extracted from cells and tissues using laser capture micro-dissection. Cañas et al. 2014 have developed a protocol for transcriptomic analyses of conifer tissue types using LCM and 454 pyrosequencing. Cryosections from unfixed frozen tissues yielded best RNAs. The complementary DNA synthesis (cDNA) was performed using an adapted protocol for conifer RNA amplification.
- Preliminary results have shown differences in number of expressed genes in 14 different cells and tissues under study.
- The NGS of 14 cDNA libraries from Oria provenance have been completed using the 454 FLX+ platform. Bioinformatic analyses are in progress.
- Transcriptomics (PINARRAY2) and Metabolomics (H1-NMR) analysis have been performed to study the growth dynamics during an annual growth cycle on samples from Sierra Bermeja (Málaga, Spain). Data analysis is also in progress.
- 37 conserved miRNA families have been identified using the miRBase v20.
- Small RNA sequencing data has been obtained from a wide range of tissues, from reproductive structures, seed and seedling tissues to xylem and phloem as well as adult needles and roots. Part of the sampled plant material was exposed to contrasting environments concerning water availability in order to address one of the objectives of the project which is related to adaptive capacity.
- A bioinformatics pipeline is being implemented for analysis of sRNA

sequencing data.

- Changes in gene expression were monitored in somatic embryos of *Picea abies* during morphogenesis under two different temperatures (18° vs. 30° C) using Illumina-based Massive Analysis of cDNA ends (MACE is a sequencing method for high resolution gene expression analysis). Distinct differences in transcriptomes were found between the genetically identical embryogenic tissues grown under the two epitype-inducing temperatures suggesting temperature-dependent alteration of gene expression during embryo development, probably due to chromatin modification. From 448 transcripts of genes coding for proteins involved in epigenetic response, 35 were found to be differentially expressed at high level under the epitype-inducing conditions. Therefore, temperature conditions during embryogenesis significantly shape transcriptional profiles. (Yakovlev et al. 2014).
- To identify differentially expressed genes during the transition from cleavage to development of a dominant embryo and functional analyses of candidate genes in *P. sylvestris* seven libraries from embryos and megagametophytes have been sequenced and the sequencing data have been analysed. Presently, expression comparisons between different samples are being conducted.
- During the first half of the project, maritime pine genotypes from controlled crosses showing contrasting physiological response to water deficit were subjected to water stress. The plants were phenotyped for various morphological and physiological traits during this treatment. Samples from different tissues are being processed during the year 2014 for small RNAs, gene expression and DNA methylation related to drought and differential temperature treatments.

Comparative Genomics (Work Package 3)

Objectives | To integrate genomic resources developed in different European and North-American model conifer species, and to carry out comparative studies for acquiring better information about their evolution and effective transfer of information to enable the study of other conifer species. Comparative mapping and gene content and order analyses will throw light on various aspects of conifer genomics like: chromosome and genome evolution, phylogenetic relationships and provide markers for GS (genome selection) based breeding.

Main results from 18 to 30 months

- COS marker sequences have been identified comparing six pine and spruce species: *Pinus pinaster*, *P. sylvestris*, *P. taeda*, *Picea abies*, *P. sitchensis* and *P. glauca*.
- A pilot exome capture, based on 60,000 probes, has been designed to analyze its usefulness in different pinaceae species using DNA from progenitors and megagametophytes of the mapping families of *Pinus pinaster*, *P. sylvestris*, *P. strobus*, *Picea abies*, *P. glauca*, *P. sitchensis* and *P. mariana* that have been selected for comparative mapping.
- The pilot exome capture contains a set of approximately 28,000 COS probes.



Pinus taeda



Picea abies



Picea sitchensis



This set targets approximately 10,000 pinaceae genes.

- Broad scale comparisons have been made between the genomes of *P. abies* and *P. glauca* to identify the factors influencing gene family size in spruce. Additionally, the genomes of three gymnosperm and eleven angiosperm species were compared to study the evolution of single-copy and large gene families.
- Whole genome data of *P. abies* (1.0 assembly) and 450 fosmid pools were aligned to 38 bacterial artificial chromosomes (BACs) from *P. glauca* to study microsynteny between both species. The percentage of coverage in the BAC scaffolds by one or several fosmid-pools varied from 0.57 to 47.48% (De La Torre & Ingvarsson 2013).

Computational Genomics (Work Package 4)

Objectives | To perform genome assembly and structural and functional annotation, systems biology analyses and an efficient and user-friendly data management. Structural annotation will identify protein coding genes, transposable elements and non-coding genes on the assembled genome. Information from transcriptome data will help in establishing transcriptional regulatory networks using softwares to link potential regulators and targets.

Main results from 18 to 30 months

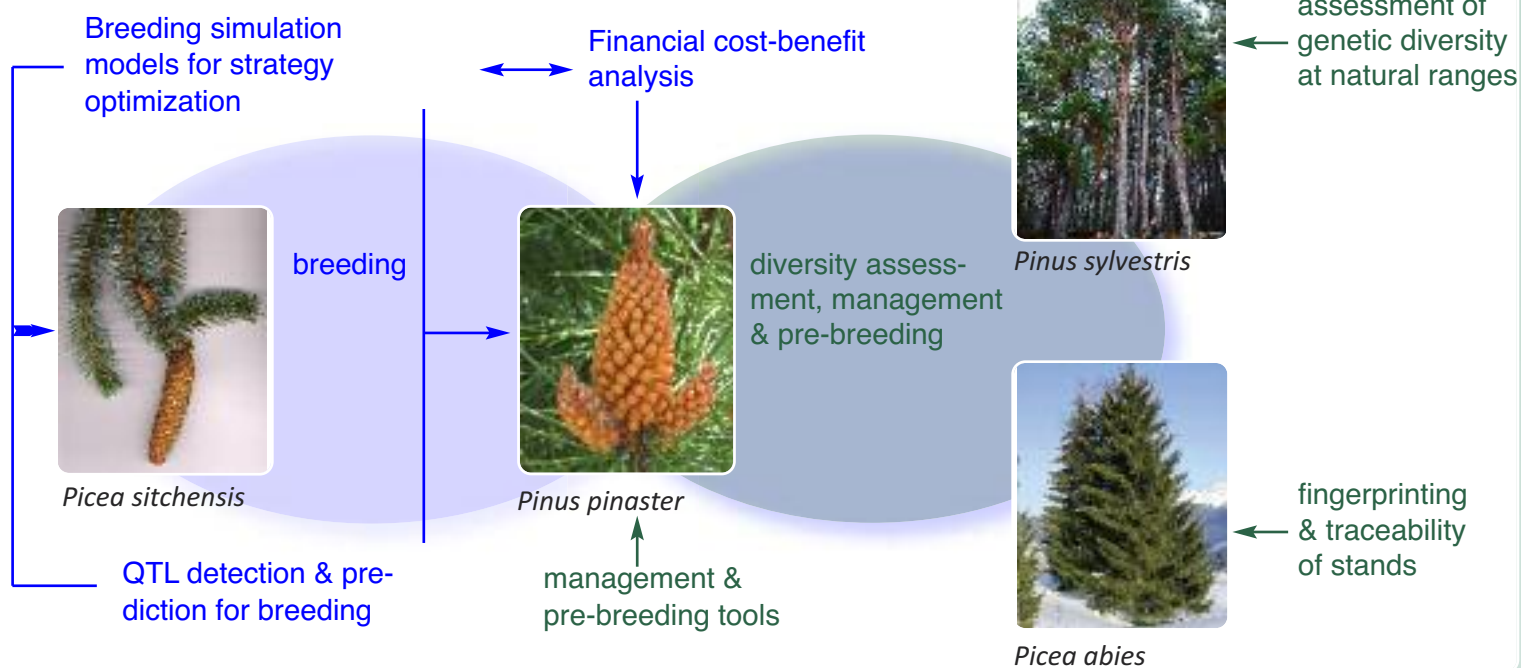
- *P. pinaster* genome assembly: assembly strategies have been benchmarked and initial assembly is in progress.
- COS markers have been developed based on available sequence data from 6 different conifer species. The analysis resulted in a set of 2,246 conserved single copy genes which for a good basis for further genotyping studies.
- Single nucleotide polymorphisms have been identified from *P. pinaster* populations. A preliminary catalog of these SNPs has been compiled. SNP identification in *P. sylvestris* is ongoing.
- Conifer oriented comparative genomics and phylogenomics online platforms are being set up.

Translational Genomics (Work Package 5)

Objectives | To identify and quantify associations between variation at the genotypic and the phenotypic levels to enable genome assisted breeding and natural resource management. Thus, translating basic scientific discoveries into applications which would result in a more accurate and efficient breeding.

Main results from 18 to 30 months

- Genomic evaluation in maritime pine- a proof of concept experiment is being developed for the implementation of genomic selection in maritime pine. Genomic selection may substantially increase genetic gain per time by reducing the reliance on progeny testing and breeding cycles. A first preliminary study



(Plomion et al. 2014) was performed to deepen the knowledge regarding genome wide diversity patterns and linkage disequilibrium in breeding populations. Using a new map merging algorithm (Endelman and Plomion 2014), first, a new 1,712 cM composite linkage map was established comprising 1,838 SNP in 12 linkage groups by bringing together three already available genetic maps. Second, a total of 2,600 informative SNPs facilitated the description of historical recombination, genetic diversity and genetic structure in 186 unrelated trees (G0) of the breeding pool for this species. Very low levels of population genetic structure was observed and no evidence was found that artificial selection caused a reduction in genetic diversity. By combining these two pieces of information, map position of 1,671 SNPs corresponding to 1,192 different loci could be located to study the pattern of genetic diversity (He) and long distance linkage disequilibrium (LD) along the chromosomes. First insights into genomic predictions were made by extending genotyping to 500 second generation plus trees (G1) with the same 2,600 markers, using BLUPs (Best Linear Unbiased Prediction) for growth and stem straightness of the G0 and G1 trees. Various statistical models were implemented to test the efficiency of genomic selection. The results of this first experiment would be a great asset in develop genomic selection strategies in maritime pine.

- A second experiment comprised the constitution of a multigenerational reference population for genomic evaluation, with reasonable effective population size (approximately of 24) for maximizing prediction accuracy yet with good diversity coverage of the breeding population. The SNP panel was enlarged by developing an additional set of 5K SNPs. Overall, the tree-generation pedigreed population comprised of 818 trees (46 G0, 62 G1 and 710 G2) and was genotyped with a 9K SNP Infinium array. A paper in preparation (Isik et al.) assesses the genomic accuracy with this new setup.
- Genomic evaluation in Sitka spruce: another proof-of-concept experiment is under development for a set of 3 full-sib families from the Sitka spruce breeding

Figure shows a schematic diagram of tasks within work package five. (Photo sources - Wiki commons): *Picea sitchensis* cone and foliage, MPK; *Pinus pinaster* - male cones, Meneerke bloem; *Pinus sylvestris* with light snow cover, Loch Garten, Nethybridge, Scotland, 57°14'44"N 3°42'09"W, 240m altitude, Lee Carson; Norway spruce, MPF.)



programme. This set comprises 1800 genotyped candidates with an expected total of 8K SNPs from a RAD approach. Phenotypic evaluations in the same set are underway for an economically important trait concerning wood density.

- In parallel to these empirical data, simulation tools are under development on two fronts. One of these models simulates haplotypes across a specified pedigree by a gene dropping approach, and allows the exploration of genomic prediction on current pedigrees with a specified genetic architecture and arbitrary number of markers. Preliminary results with this model applied to the maritime pine pedigree suggest that, compared to pedigree information, current marker densities provide a gain in accuracy similar to that achieved by knowing the true genomic ancestry. These results are part of an article in preparation that will include other breeding programs and that aims at assessing the scope of genomic evaluation across forest tree breeding programs. Another on-going model development that is more generic concerns the assessment of the different factors in the design of the reference population that affect accuracy of genomic predictions.
- A financial cost-benefit analysis tool is under development. This is a parametric simulation tool that will provide a cost-benefit assessment of traditional and genome-based evaluations by taking into consideration all breeding steps in a typical improvement cycle.
- Assessment of genomic diversity at the natural range scale and redefining core collections- A detailed compilation of existing nuclear microsatellites (nSSR) and SNPs has been performed for Scots pine. This comprises 14 easily scorable nSSR (in two multiplexes of eight and six markers) that display high polymorphisms and low null allele frequencies, and all published SNP information related to the species.

In addition, an analysis of the distribution of neutral genetic diversity using nuclear and chloroplast SSRs was performed. Concerning the biological sampling for the species, a gap analysis was performed to identify areas for which samples are lacking and results were subsequently used to get additional collections. In parallel, an inventory for the species reference material from French natural genetic resources has been created to extend the coverage of the core collection to a wide European level. This is essential due to the proven fact that Scots pine exhibit phenotypic differentiation between populations, the effects of century-long cultivation, and the expected environmental changes at the margins of the distribution range.

- Fingerprinting and traceability of genetic resources in Norway spruce- this on-going study has already undertaken two exploratory steps, one that concerns the selection of study sites for the species in afforested stands, and another in evaluating the genotyping possibilities, including a recently developed SNPs chip of a few thousands markers. Biological samples are underway.
- Polymix breeding and paternity analysis- the usefulness of pedigree reconstruction in progenies of polymix crosses has been assessed in a maritime pine case study. A first analysis with ca. 60 SNPs allowed a full pedigree

reconstruction in 500 progenies. Reconstructed pedigree was subsequently used for genetic evaluation, as genotyped progenies were also phenotyped for various growth traits (diameter, height), wood quality (stem straightness, spiral grain, wood density) and adaptive traits (water use efficiency). Based on this data set, different strategies derived from the use of polymix were evaluated in terms of genetic gains and compared to standard method without paternity analysis.

Integration & Dissemination (Work Package 6)

Objectives | To integrate efforts in this project with similar large-scale initiatives e.g. in North America and Canada and to ensure efficient transfer of knowledge and applied outcomes of the project to other research groups as well as to organisations involved in conifer breeding and management.

Main results from 18 to 30 months

- The ProCoGen partners are working closely with US, Canadian and other European initiatives. There has been transfer of knowhow and interactions at training workshops, meetings, lab visits and through mailings.
- The first dissemination workshop was held in November 2013 in Latvia.
- The first internal staff exchange programme has also been accomplished.
- The second training workshop was held in Alcala, Spain.
- The procogen blog has attracted 1,840 viewings from 57 countries till date.
- ProCoGen partners have actively participated in meeting of other EU projects, symposia, and international events organised by IUFRO, EFI, COST Action, Plant and Animal Genome conference, Bioinformatic conference, Post transcriptional gene regulation (plant biology meeting), *In vitro* propagation, etc.

Suggested reading and references

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Events

Training Workshop | The second hand-on training workshop was held in the University of Alcalá de Henares, Spain from 19th - 21st Feb. 2014. The theme was "Conifer Functional Genomics: analysis of gene networks involved in conifer adaptation". Fifteen invited speakers from Europe, Canada and the USA, and about 40 participants on an average/day attended this event. The audience comprised of project members, local researchers and students. The topics extended from the latest technological advancements in sequencing, and bioinformatic tools available for gene and genome annotation, to concepts of epigenetics, epigenomics and applications of functional genomics. Lectures based on the latest advancements in functional genomics of conifers especially from the ongoing European and Trans-Atlantic initiatives were received with utmost interest. (Most of the lectures can be downloaded from the homepage)



Dissemination Workshop | The first dissemination workshop was held in Riga, Latvia from 5th - 6th of Nov. 2013. It was an effort to interact with young researchers and scientist from EU countries not involved in this project, with special focus on the Baltic region. The main aim was to bridge the knowledge gap and introduce young researchers and scientists to the state of the art research and application of new technologies in developing genomics resources. The workshop offered an interactive platform for encouraging the participants to exchange their views, ideas and plans in the field of tree genomic research.



The workshop was attended by 26 people on an average, from 10 different countries. The workshop was organized by BFW (Austrian ProCoGen partner), with the support of Dr. Dainis Rungis from LVMI (Silava), Latvia, whose contribution was vital to the success of this workshop. One Trans-Atlantic and three European speakers (from ProCoGen partner institutions) were invited to present at this workshop. Each was required to give two presentations of about 50 minutes each, one comprising of more theoretical material and the second highlighting recent developments, demonstrating research updates and applications. The lectures can be downloaded from the project homepage.

The second dissemination workshop will be held in Kámoni Arboretum Szombathely, Hungary from 1st - 3rd Sept. 2014, with the main focus on central-eastern European region. The theme will be "Genomics and conservation of genetic resources". This workshop is being organised by BFW in cooperation with the FORGER EU FP7 project (The programme and invitation can be downloaded from the project website).

Publications

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Photo sources page 1 (wiki commons): Bugaboo forest fire: Mark Wolfe/FEMA; Unter der Rinde: Smial; Dry earth on Sonora desert: Tomas Castelazo; Logging in Finnish Lapland: Green-peace Finland
 Photo sources page 2: *Picea abies*: Leo Michels; *Picea sitchensis*: Gerald and Buff Corsi@California Academy of Sciences.
 Photo sources page 3: *Pinus pinaster*-male cones: Meneerke Bloem; *Pinus sylvestris* var. *hamata* foliage and cones: Bulgaria;
 Photo sources page 4 (wiki commons): *Pinus sylvestris*: Jonathan Kington; *Pinus pinaster*: William Scot;
 Photo sources page 5 (wiki commons): *Pinus taeda*: US NPS photo; *Picea abies*: MPF; *Picea sitchensis*: Roland Tanglao;
 Photo sources page 6: *Pinus pinaster* plantation, Didier Bert, INRA, France;
 Photo sources page 8: Polytent nursery_pnie breeding: Didier Bert.tif
 Photo sources page 10 and 11: BFW_ProCoGen

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- Canada:** Université Laval (Ulaval)
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