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STSM
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Controlled pollination technique and genetic variability of Pinus mugo x Pinus sylvestris hybrids

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FINAL REPORT

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Abstract - Context and scientific objectives

Population structure of small marginal populations acting together with environment and climate events more extreme than at the species core distribution may create conditions for overlapping of flowering phases that normally are desynchronized. Shall the reproductive phenologic behaviour of the trees find appropriate conditions intra-specific crosses but also inter-specific crosses may occur and hybrid progenies may happen.

The study of hybrid swarms evolution is most important for conservation of genetic resources of forest small and marginal populations.

The hybrid swarm population blurs between the parent taxa and may interbreed and backcross with its parent types giving origin to highly variable population with the genetic and phenotypic characteristics of individuals ranging widely between the two parent types.

The genetic material is located in non-nuclear structures, in chloroplasts and mitochondria. Some authors argue that both genomes are inherited in paternal (cpDNA) and maternal (mtDNA) line and are not the subject of sexual recombination (Wagner *et al*, 1987). Molecular evidence based on parallel analysis of the maternally (mtDNA) and paternally (cpDNA) inherited DNA can confirm the hybrid nature of individual trees of the swarm.

Genetic variability of cpDNA in conifers is known, but species-specific mtDNA markers are not yet known. However, from introgression studies we can say that seeds could be putative hybrids.

Introgression results in a complex mixture of parental genes, while simple hybridization results in a more uniform mixture, which in the first generation will be an even mix of two parental species. So with a simultaneous analysis of the respective cpDNA region using needles of a given tree along with megagametophytes and embryos of individual seeds, and if there are differences between megagametophytes and embryos (different paternal DNA) we can say that there is a putative hybridization.

(Kormutak *et.al,* 2006)

On the other hand, in the future, we consider the use microsatellites to study more differences between the hybrids trees for characterization of Portuguese MaP FGR of *Pinus pinaster.*

On controlled pollination programs for conservation of genetic resources or resistance to pest and diseases ongoing on RePhrame project (http://www.rephrame.eu/) it is very important to assess if hybrid swarms are being developed.

Purpose of the STSM

The STSM was focused on hybrid screening through PCR-RFLP of DNA extracted from needles of maternal trees, embryos and female gametophytes of seeds originating from artificial pollination *Pinus mugo* × *Pinus sylvestris* since this technique is optimised to check parentality.

For the objective of bring knowledge to INIAV for *P. pinaster x P. halepensis* hybrids screening this STSM was very useful.

The use of microsatellites technique was discussed and I could profit from the team experience on microsatellites. At the host institute we have used PCR-RFLP approach instead of formerly planned microsatellite DNA markers. The main reason for this

preference was much higher efficiency of cpDNA markers in verification of hard pine interspecific hybrids than with microsatellites. Still other reason is that method is not so much expensive as in case of microsatellite DNAs.

Methods and Results obtained at the Institute of Plant Genetics and Biotechnology SAS in Nitra

Methodology

In presented work we have analysed offspring seeds obtained by the artificial hybridization between *P.mugo* x *P.sylvestris* done last year, in Nitra, Slovakia.

We started to extract total DNA from seed tissues like endosperm and embryo using the CTAB method (Sbs – Nucleic Acids Isolation and Purification; Genetech Co., Lda) and a specific protocol for DNA isolation from seed of *Pinus* species by Xiao-Ru Wang, 2000 (Fig1).

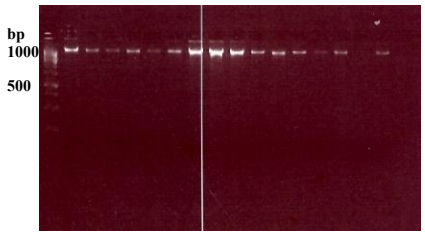

Fig1- Total DNA isolated from hybrid embryos *P.mugo* x *P.sylvestris*

The *trn*V-*trn*H primer specie-specific for cpDNA was PCR amplified using the primer pair which consisted of 5'-GCTCAGCAAGGTAGAGCACC-3' and 5'-CTTGGTCCACTTGGCTACGT-3' (Parducci and Szmidt.1999).

DNA amplification was performed at 94ºC for 4 min followed by 35 cycles at 93ºC for 1 min, 56ºC for 1 min and 72ºC for 2min. The last strand elongation at 72ºC was allowed an additional 10min. To confirm successful amplification of the cpDNA region, 5µL of the PCR products were separated by electrophoresis in 1% agarose gel.

PCR product were digested with the restriction enzyme *Hinf* I, which has been found to discriminate the cpDNA of *P.sylvestris* from that of *P.mugo* (Kormutak et al, 2002). The generated fragments were fractioned electrophoretically in 8% polyacrylamide gels. (Figs.2 and 3)

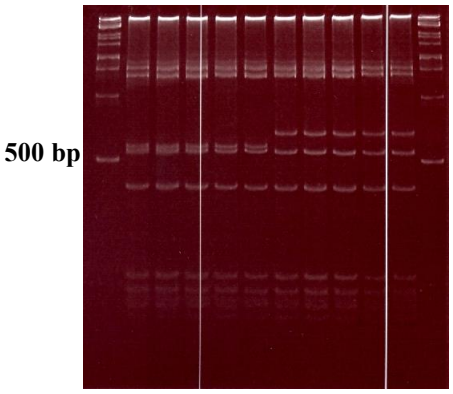

Fig. 2- cpDNA*trn*V-*trn*H/*Hinf* I restriction profiles of *P.mugo* and *P.sylvestris* needles; m- size marker; 1-5- *P.mugo*; 6-10 - *P. sylvestris*

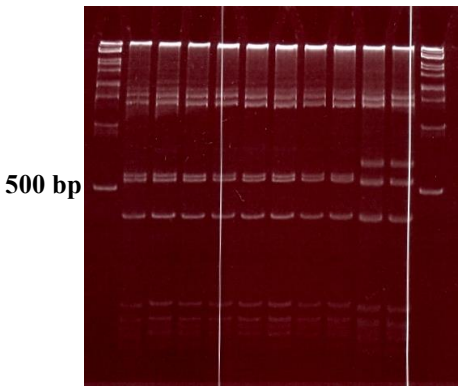

Fig. 3- *trn*V-*trn*H/*Hinf* I based verification of hybrid seeds *P.mugo* ×*P.sylvestris*; m- size marker; 1,2 - needles and megagametophyte of *P. mugo* mother tree; 3-9 –hybrid embryos of the same tree exhibiting *P.mugo* haplotype (3-8 lanes, non-hybrid seeds) and *P.sylvestris* haplotype (lane 9, hybrid seed). Lane 10 – paternal tree *P. sylvestris*

Results

The species-specific nature of *trnV-trnH*/*Hinf*I restriction profiles of cpDNAs is based on differential occurrence of the 680 bp fragment in *P. mugo* (Fig. 2, lanes 1-5) and 700 bp fragment in *P. sylvestris* (Fig. 2, lanes 6-10). The 670 bp fragment is commonly shared by the parental species *P. mugo* and *P. sylvestris*.

Using this cpDNA marker, we have made an attempt to verify hybridity of the seeds from controlled pollination *P. mugo*× *P. sylvestris*. In total, 7 seeds were subjected to analysis but only one of them was proved to be the hybrid. The results of this analysis are illustrated in Fig. 3.There are size markers on both sides of the polyacrylamide gel along with restriction profiles of the maternal tree *P. mugo* (lane 1 – needles, lane 2- female gametophyte), tested embryos (lanes 3-9) and the paternal tree *P. sylvestris* (needles). Only one embryo (lane 8) exhibits restriction profile of the paternal tree *P. sylvestris*. Owing to the paternal inheritance of cpDNA in conifers (Wagner et al. 1987), this may be taken as an unequivocal evidence of its hybrid nature.

It is expected that the same methodology will be applied in detection of the putative hybrid seeds between *P.pinaster* x P. *halepensis* in Portugal where intercross between these species are ongoing for further studies oriented for Pine wood Nematode-PWN resistance.We plan to look for the species-specific markers of both mitochondrial DNA and cpDNA for these species to make a paralleled analysis of these markers in the putative hybrid individuals. Also, further studies are planned oriented towards characterization of Portuguese MaP FGR in *Pinus pinaster*.

Material from Portugal- due to the exiguous number of cones alive by the second summer after controlled pollination no hybrid seeds between *P.pinaster* x *P. halepensis* in Portugal was not taken to the host lab since it has been decided to save the existing material for RePhrame research.

Institutional added value and future collaboration

This STSM has been an added value for me and for INIAV on the issue of *Pinus* sp hybridization namely on DNA screening.

The STSM opened up opportunities for collaboration both on the level of institutions and scientists.

The scientific exchange among INIAV and Institute of Plant Genetics and Biotechnology is facilitated and it is expected to go beyond the concrete subject of this STSM in the fields of projects candidatures, improving of techniques, PhD and post-doc hosting, etc.

Acknowledgements

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