FPS COST Action FP1202

Strengthening conservation:
a key issue for adaptation of marginal/peripheral populations
of forest trees to climate change in Europe (MaP-FGR)

SHORT TERM SCIENTIFIC MISSION REPORT

Learning molecular techniques for genetic characterization of forest tree populations

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STSM period: 1 March 2016 to 10 April 2016:

COST STSM Reference Number: COST-STSM-FP1202-32290

COST STSM Reference Number: COST-STSM-FP1202-33696 (STSM extension)

Abstract

The purpose of this scientific training was the acquisition of molecular techniques and methods for genetic characterization of Montenegrin forest tree populations.

During the first part of the training (1 – 31 March 2016), plant material from 3 Montenegrin populations of Pinus heldreichii were processed for genetic characterization with nuclear and chloroplast microsatellite markers (nSSRs and cpSSRs). The obtained data was then analyzed with genotyping and statistical softwares. By the end of March, the newly arrived plant material of 3 populations of Abies alba and extracted DNA of three populations of Pinus peuce were processed according to learned methods and procedures, and the work continued and was finished in the 10 days long STSM extension period (1 - 10 April 2016).

All protocols, techniques and methods learned will be implemented in the laboratory of Forestry department of the Biotechnical faculty of Montenegro. The results of Pinus heldreichii and Abies alba will be used for comparison studies with the already characterized samples present at IBBR data collection and a joint publication is planned. In the case of Pinus peuce, which genealogy has not been studied yet, the characterization conducted will serve as a basis for future studies.

I. Introduction

The previously established collaboration between the Institute of BioResources and BioSciences (IBBR) of the National Research Centre, Italy, and Biotechnical faculty (BTF), University of Montenegro through bilateral project: Tree fragmented populations from refugial areas: the anfiadriatic connection, has already underlined the necessity and urgency to genetically characterize Montenegrin tree populations, especially those at the rear edge of their distribution.

Montenegrin forests possess extremely valuable genetic resources (Lazarević, 2012). Genetic characterization of Montenegrin forest trees populations, as it is a case with Balkans in general, especially of those species with geographically limited distribution has not been conducted yet. Therefore, their genetic characterization would be crucial for their historical relevance and also because they can represent the source of potentially adaptive genetic variation to cope with ongoing climate change (Hampe & Petit, 2005).

The Balkans are considered one of the major refugial areas during past glacial cycles, which hosts the southernmost Norway spruce (*Picea abies*), silver fir (*Abies alba*) and Bosnian pine (*Pinus heldreichii*) populations, besides other understudied species with locally highly fragmented distribution (e.g. Black pine, *Pinus nigra*) or extremely limited distribution (e.g. Macedonian pine, *Pinus peuce*). Given the richness of forest trees diversity and biodiversity, it is necessary to make major contribution to their study and conservation.

This research training primarily aimed at learning molecular methods for genetic characterization of forest tree populations in close proximity with refugial area with nuclear and chloroplast microsatellite markers (nSSRs and cpSSRs). The analysis was focused on most relevant species such as *Pinus heldreichii* (Christ.), *Abies alba* (Mill.) and *Pinus peuce* (Gris.). Apart from the highly fragmented and isolated species Pinus peuce, the other two species have already been studied by the host institution, IBBR, and this guaranteed a number of already optimized laboratory protocols and procedures and sampled populations providing a good starting point to efficiently add new populations to existing datasets.

II. Objective of the STSM

The main objective of this research training is the acquisition of molecular techniques, methods and optimized protocols for genetic characterization of marginal/peripheral forest tree populations and data analysis, which will be subsequently implemented in the laboratory of Forestry department of the Biotechnical faculty of Montenegro. In addition, significant data and results obtained will be used for the future work and added to the already existing dataset of IBBR at CNR.

This procedure includes:

1) Genetic characterization with already available molecular markers (nSSRs and cpSSRs) of 3 Montenegrin populations of *Pinus heldreichii* and 3 populations of *Abies alba*. For 3 populations of *Pinus peuce*, tests for transferability of molecular markers (cpSSRs and nSSRs) from other *Pinaceae* were conducted;

2) Basis of bioinformatics - data analysis using genotyping and statistical approaches;

3) After data collection from fragments analysis and scoring, newly genotyped *Alba abies* and *Pinus heldreichii* populations will be added to already existing datasets.

III. Links with Cost Action FP1202 MaP FGR

As a part of the Cost Action FP1202 MaP FGR – Strengthening conservation: a key issue for adaptation of marginal/ peripheral populations of forest trees to climate change in Europe (MaP-FGR), this research training consisted in genetic characterization of populations of *Pinaceae* species – *Pinus heldreichii*, *Abies alba* and *Pinus peuce*. As the host laboratory possess a significant research experience and data collection for the species of P. heldreichii and A. alba, in addition to the learned molecular techniques, methods and bioinformatics' tools, it also represented an important work during which new data and results on the understudied Montenegrin populations and species have been produced. The data from the transferability tests of markers from other *Pinaceae*, conducted for a fragmented coniferous species with limited natural range within the Balkans of *P. peuce* will be used for the future work on this species and dataset creation. These data represent the basis of the conservation and long-term survival of the studied species.

IV. Materials and methods

Plant material

The training focused on genetic characterization of the tree species sampled in Montenegro which include:

- 3 populations of *P. heldreichii*, represented by ca. 50 geo-referenced individuals per populations (165 samples in total). The selected populations were collected on Mt. Prekornica, Mt. Orjen and Mt. Kučka korita-Hum Orahovski. Genetic characterization was conducted with 12 nuclear and 8 chloroplast microsatellite markers (nSSRs and cpSSRs).

- the sample of 108 individuals from 3 population of *Abies alba* (ca. 35 geo-referenced individuals per population, collected from Mt. Prokletije loc. Visitor, Mt. Prokletije loc. Babino polje-Bogićevica, Mt. Bjelasica). Genetic characterization was conducted with 14 nSSRs and 3 cpSSRs.

- the working material for *Pinus peuce* consisted in extracted DNA samples from 3 populations (around 45 geo-referenced individuals per population, sampled on Mt.Prokletije loc. Bogićevica, Mt. Prokletije loc. Visitor, Mt. Zeletin) and tests for markers transferability from other *Pinaceae* have been conducted with 40 nSSRs and 11 cpSSRs markers.

The characterization included the following procedures:

DNA extraction and quantification

DNA extractions from plant tissue (ca. 50 mg of dried needles) were performed using NucleoSpin 96 Plant II protocol (MACHEREY-NAGEL, GmbH & Co. KG, Germany). This

procedure includes the use of the 3 mm diameter steel bead for each well of the 96-well plates, which eases the cell disruption. Plates were frozen at -80˚ C overnight before 2 cycles of 1-min disruption at 25 Hz using a Mixer MillMM300 (Retsch, Germany). The following steps consist in cell lyses using appropriate buffers (SDS method) with the addition of Rnase A, and DNA binding to silica membrane. After the membrane washes, DNA elution is performed with RNAse free water.

The extracted DNA concentrations were measured using a spectrophotometer NanoDrop ND 1000 (Thermo Scientific, Wilmington, USA).

Multiplex PCR with nuclear and chloroplast microsatellite markers

Considering the previous experience of IBBR in genetic characterization of tree populations, already designed primer pairs have been used in multiplexes for both *P. heldreichii* and A. alba. Multiplex PCR reactions, carried out using Type-it Microsatellite PCR kit (Qiagen, GmbH, Hilden, Germany) and following manufacturer instructions, were conducted with a PTC100 thermal cycler (MJ Research, San Francisco, CA, USA). The optimal PCR profiles have been set up according to the Type-it Microsatellite PCR Kit (Qiagen) instructions, depending on the primer mix. This includes an initial activation step, 5 minutes at 95˚ C, in which HotStarTaq Plus DNA Polimerase is being activated. This polymerase is provided in an inactive state in the Type-it Multiplex PCR Master Mix. The optimal annealing temperature was set up at 57˚ C.

The markers were multiplexed by taking into account their size ranges.

Nuclear variations were analyzed for 12 microsatellite loci for *Pinus heldreichii* and 14 for *Abies alba*. 8 chloroplast markers were analyzed for *P. heldreichii* and 3 for *A. alba*.

Single PCR reactions

11 chloroplast and 40 nuclear, previously developed markers were analyzed for the transferability from other *Pinaceae* to the understudied populations of *P. peuce*.

Using different PCR conditions for single PCR reactions – a touchdown 60-50 PCR profile with a variable annealing temperature (10 cycles at 60˚ C, decreasing one degree in temperature per cycle, following 25 cycles at 50˚ C) which ensures a major specificity and sensibility in amplification. Single PCRs have been performed for every marker and carried out using GoTaq DNA Polymerase (Promega, Medison, Wisconsin, USA), according to manufacturer instructions.

Fragment analysis

Screening of PCR products was performed by a 3500 Genetic Analyzer (Applied Biosystems, Inc., Foster City, USA), using LIZ-500 as an internal size standard (Applied Biosystems, Foster City, USA). To evaluate SSR polymorphism, samples were genotyped with SSRs that exhibited high-quality amplification and clear microsatellite peaks of the expected size.

Analyses for missing data has been repeated and every relevant data was included into the evaluation dataset.

Besides molecular techniques, important bioinformatics' methods have also been used:

Genotype Scoring and Data Analyses

The obtained chromatograms were analyzed by allele binning and calling using GeneMarker ver. 2.4.0 (Holland and Parson 2011), a program able to analyze genotype data from microsatellite analysis.

The assessment of levels of genetic diversity and structure for *P. heldreichii* and *A. alba* was conducted in reference to the present pool of the SSR data using the Bayesian clustering approach - software STRUCTURE Ver.2.3.4 (Falush et al. 2007; Pritchard et al. 2010) and STRUCTURE Harvester (Earl and vonHoldt, 2012. The STRUCTURE and the STRUCTURE Harvester software are used to study the the genetic structure and determine the optimal number of clusters among the studied populations (Pritchard et al. 2000; Falush et al. 2003; 2007).

V. Results and discussion

The most important outcome of this scientific mission is a successfully gained knowledge on molecular techniques and procedures for genetic characterization of tree populations, which will be implemented at BTF. In addition, a significant number of data have been obtained. The data analysis was conducted on the basis of the already available data pool for the species of *P. heldreichii* and *A. alba,* and the final data will be added to the corresponding dataset.

The preliminary results on *P. peuce* will be analyzed in future, as more studies and on more populations need to be conducted. The expansion of this dataset could be an important goal for the future projects, as this species is genetically unexplored and highly isolated and fragmented. This dataset and further work on genotyping of *P. peuce* populations would provide new insights into their genetic patterns and variability.

VI. Conclusion

As a result of the 40 days long visit to IBBR, main techniques for genetic characterization of tree populations have been acquainted and a significant data set has been generated. The data obtained will be used for the already existing datasets for *P. heldreichii* and *A. alba* at IBBR, as well as for some of the current and future projects of both CNR and BTF, where joint publications are planned.

I also consider very important the approval of the STSM extension request, as I was able to repeat the entire molecular procedure on the populations of *A.alba* and also on the populations of *P. peuce* and therefore to conduct a short, but new research, which should be continued with the prospective of very significant results.

In the end, I appreciate very much this opportunity to visit and work in IBBR's laboratories and learn from Dr. Vendramin's team members who possess expertise in the field of tree

genetics. I am positive that this professional training will have a significant impact on my future work, as well as on the future joint studies of mentioned and other forest tree species, especially of those important but understudied Montenegrin populations. As a part of a much broader objective, continuous work on the genetic characterization will contribute to the conservation of genetic resources and long-term survival of the species.

List of contents