LIFE4FIR – Project LIFE18 NAT/IT/000164

"Decisive in situ and ex situ conservation strategies to secure the critically endangered Sicilian fir, *Abies nebrodensis*"

""Implemented procedure to quickly determine the genetic origin of seedlings. Dissemination of our results to Natural Park Managers at the end of the Project" - Action C2.

Istituto per la Protezione Sostenibile delle Piante
Consiglio Nazionale delle Ricerche

Consiglio Nazionale delle Ricerche
Istituto per la BioEconomia
Dipartimento di Scienze Bio Agroalimentari

REPUBBLICA ITALIANA
REGIONE SICILIANA
ASSESSORATO REGIONALE
DELL'AGRICOLTURA, DELLO SVILUPPO RURALE
E DELLA PESCA MEDITERRANEA

Parco delle Madonie

UNIVERSITÀ DEGLI STUDI DI PALERMO
SAAF DIPARTIMENTO SCIENZE AGRARIE ALIMENTARI FORESTALI

UNIVERSIDAD DE SEVILLA

TABLE OF CONTENTS

"Implemented procedure to quickly determine the genetic origin of seedlings. Dissemination of our results to Natural Park Managers at the end of the Project"

1. Introduction

Given that seedlings from nursery are to be used in future reforestation actions, it is important to disentangle their genetic origin to differentiate pure *Abies nebrodensis* seedlings from putative hybrids with other alien fir species. The procedure consists of three steps: DNA collection, sample sequencing and bioinformatics analysis. Open array was the technique of choice because of its high capacity to detect differences even between closely related individuals.

2. STEP 1- Material collection and DNA extraction.

Collect young leaves of the seedling to be studied. Leaves should be dry and without signs of infection by pathogens. About 5-6 leaves are sufficient for genetic characterization. Introduce these leaves in a porous bag (tea bag type) and label the bag with the data of the seedling. Include the bag in another plastic bag in which enough silica gel has been placed to surround the sample bag. Close the plastic bag tightly. The silica gel has a moisture indicator, so it changes color when its ability to dry has been lost. If this occurs, the silica gel should be replaced with new silica gel. DNA extraction can be done easily using the protocol shown below, although it can also be contracted with any genomics company.

Protocol for DNA extraction: Isolate DNA by using the NucleoMag Plant kit (Macherey-Nagel, Germany) according to the manufacturer's protocol but with the following modifications.

1) Add a volume of 650 uL of the lysis buffer MC1 with RNAse to each sample.

   Centrifuge the samples at a centrifuge (eg. Allegra X-12 Beckman Coulter, USA) for 40 minutes at a full speed (3750 g) to obtain clear lysates.

2) Use of the RAW buffer instead of the wash buffer MC4 and the omission of the wash with MC5 buffer. This latter step of the protocol is necessary to remove traces of ethanol, but negatively affect the quantity of DNA concentrations of *Abies nebrodensis* samples. Instead, samples have to be kept at room temperature for 10 minutes to ensure that traces of ethanol were volatilized.

3) Separate the magnetic beads for 5 minutes against the side of the wells by placing the Squarewell Block in the NucleoMag SEP magnetic separator.

4) Measure the DNA concentration with Qubit 3.0 (Thermo-Fisher, USA) and assess the purity of DNA by measuring the 260/280 and 260/230 ratios with a NanoDrop DS-11 spectrophotometer (NanoDrop Technologies, USA). Store the DNA extracted at −20ºC.

3. STEP 2-Sampling sequencing

DNA was used to genotype *A. nebrodensis* individuals using the PCR-based OpenArrays technology following manufacter's protocol (Thermofisher, USA). Based on previous genomic data (Balao et al., 2020), we designed an OpenArray assay with 120 single nucleotide polymorphisms (SNPs) composed by information-rich SNPs in samples of *A. nebrodensis*, *A. alba* and *A. cephalonica*. In particular, 20 SNPs were selected for their power to discriminate putative hybrids between *A. nebrodensis* and the other two Abies species present in the natural population. The remaining 100 SNPs were selected for the paternity test for *A. nebrodensis* based on their high diversity.

This technique can be contracted with the different companies that currently exist in the market and that work in genomics.

4. STEP 3-Bioinformatic analysis to carry out the paternity test

We recommend the use of the software COLONY to conduct paternity tests on the seedlings in order to determine the rate of outcrossing, inbreeding and self- fertilization, as well as to assess the eventual hybridization events due to pollen coming from A. alba and A. cephalonica. Seedlings with a putative hybrid origin or with an uncertain genetic origin must be investigated by a PCA and a DAPC, using 95 of 120 loci (including 18 out 20 selected for this purpose) present in *A. nebrodensis*, *A. cephalonica* and *A. alba*. The sequencing of the 120 loci are available at https://idus.us.es/handle/11441/133288.

Bioinformatic analysis can be contracted with the different companies that currently exist in the market and that work in genomics.