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Innovative In Situ and Ex Situ Conservation Strategies of the Madonie Fir *Abies nebrodensis*

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Abstract: *Abies nebrodensis* (Lojac.) Mattei is an endemic species of the north-west of Sicily located in an 84 ha area in the Madonie Regional park. The current population is limited to 30 relic adult trees and a fluctuating number of juveniles of natural regeneration. The species is defined as “Critically Endangered” in the Italian list of threatened plants and is classified as CR-D in the 2000 IUCN Red List of Threatened Species. This article reports the key action undertaken by the LIFE4FIR project aimed at preserving *A. nebrodensis*, and the results obtained so far in three years of activity. OpenArrays SNPs genotyping revealed a high rate of inbreeding in the natural population and that the adult trees are genetically related. Controlled cross-pollination was consequently performed to increase the genetic variability of the progeny. Outbred offspring are currently being grown in the nursery. Reforestation has been planned by using 4000 selected outbred seedlings in 10 areas within Madonie Park to create re-diffusion cores. Support and protection of the relic population have been implemented through regular phytosanitary surveys, as well as new fencing and video surveillance systems against grazing and wild herbivores. A seedbank and cryobank for the long-term germplasm conservation have been established.

Keywords: Mediterranean fir; cryobank; seed bank; reforestation

1. Introduction

The main cause of loss of biodiversity is due to indiscriminate human intervention and the non-sustainable use of natural resources, which is largely responsible for climate change, habitat loss and fragmentation, pollution, introduction of alien species and over-exploitation. Around 46% of the forests in Europe are dominated by coniferous trees, where firs (Pinaceae) are represented by 12 taxa [1,2]. *Abies nebrodensis* (Lojac.) Mattei, which is the subject of this paper, is a well-known and most representative endemism of Sicily and currently one of the rarest conifer species in the world. Its residual population reveals the consequences of the over exploitation of natural resources and its impact on biodiversity and habitat degradation.

The relic population of *A. nebrodensis* (Lojac.) Mattei occupies a very small area of 84 hectares, at between 1400–1650 m a.s.l., located in the Madonie Regional Park in the

north-west of Sicily [3] (Figure 1). The habitat is listed as ‘Apennine beech forest with *Abies alba* and beech forests with *Abies nebrodensis*’ (Code 9220 in Habitat Directive) in the Annex I of the Habitats Directive and is part of the Natura 2000 network (habitat 9220*, ZSC Ita 020004, ZPS 020050) (Figure 2). Very little is known about the origin of this species. It could be easily confused with *Abies alba* and shares morphological characters with the neighboring species *Abies cephalonica* and *Abies numidica* [4]. However, very specific phenotypic characteristics, such as the smaller size, glabrescent branchlets and non-pectinate stiff leaves, mean that it can be differentiated from the other taxa.

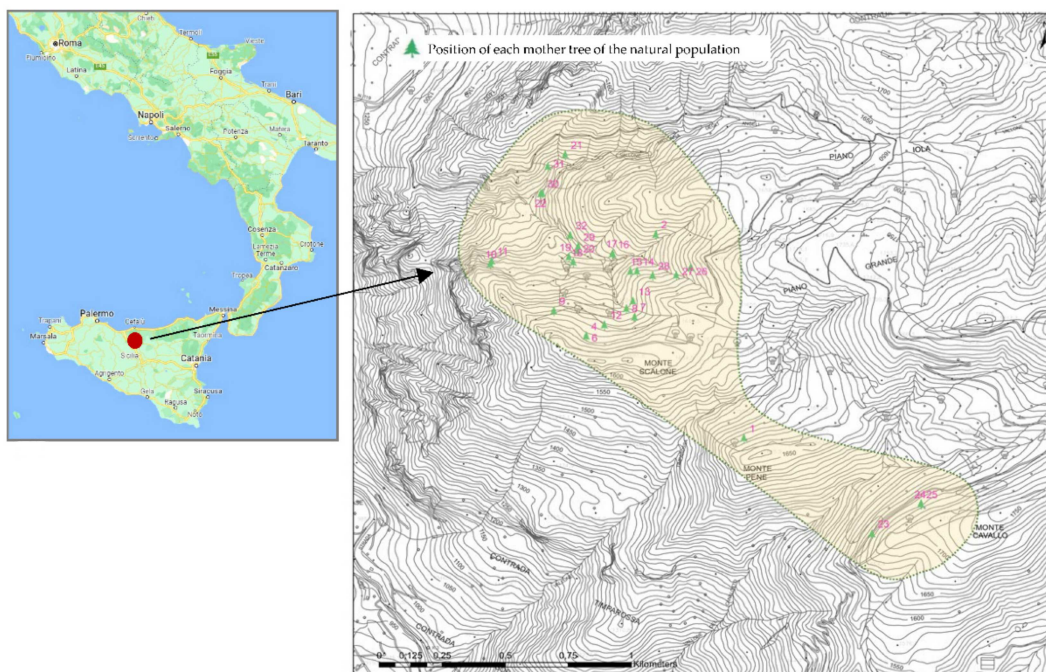


Figure 1. Localization of Madonie park in the Sicily region (left) and map of the *A. nebrodensis* natural population (right). Position of each 30 adult trees is reported in a 1:10,000 Sicily regional map based on the Gauss-Boaga projection.

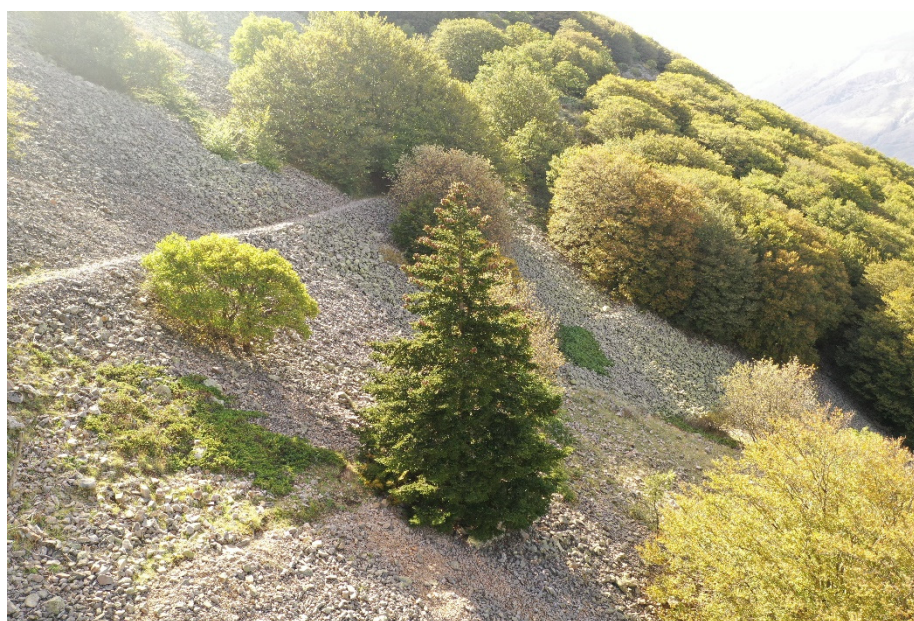


Figure 2. Mother tree no. 8 in the natural habitat of *A. nebrodensis*.

Many authors have hypothesized that *A. nebrodensis* originated from the southern population of *A. alba* during the post-glacial periods [5–8], but also its appearance through past hybridization events between *A. alba* and *A. numidica* (Algerian fir) has been suggested [4,9]. Morandini (1969) [5] reported that *A. nebrodensis* trees were frequently encountered on the Madonie mountains during the 17th century, until the population declined drastically due to anthropogenic pressure. In fact, the wood of the Madonie fir was widely exploited to build roof beams of buildings in nearby towns, and also for firewood [3,10]. Self-pollination and inbreeding that often occur in *Abies* taxa, along with the dispersed distribution of the trees and the Wahlund effect due to the weak gene flow, led to a very fragmented endangered relict population. Today there are only 30 trees left [3,5,10,11], whereas 500 would be needed to ensure a long-term viable population, as suggested by Frankel et al. [12]. Moreover, this small and isolated population with a low level of heterozygosity could be more vulnerable to adversity. *Abies nebrodensis* was thus categorized as “Critically Endangered” on the Italian list of threatened plants [13] and classified as CR-D in the 2000 IUCN Red List of Threatened Species (<http://www.iucn.org/redlist/2000/> (accessed on 27 June 2022)).

The preservation of this species has been faced in the last decades with a series of projects aimed at both in situ and ex situ conservation [14,15], among these the LIFE00/NAT/IT/007228 (2000–2004). Efforts to increase the population of *A. nebrodensis* have been implemented by experimental plantation of seedlings obtained from open pollinated seeds in some localities of Madonie. A system of fences was also installed to protect the habitat, reduce soil erosion, and support natural regeneration [16].

In the management of limited number of effective individuals, data about genetic structure are very helpful to improve or develop new conservation strategies. Several studies have explored the genetic diversity of the Sicilian fir and its interspecific relationships by molecular markers such as allozymes [6,11,17], RAPDs from the nuclear genome of diploid cells [11], PCR-RFLP [9,11] and microsatellites from cpDNA [9,18]. Such studies have reported a high genetic distance among individuals, which is rarely reported in conifers. Although studies contributed to the knowledge of the genetic structure of the residual population of *A. nebrodensis*, their findings were not used to implement measures for increasing the genetic diversity of the species.

In 2018 the LIFE4FIR project was developed to put in place an effective strategy for improving the conservation status of *A. nebrodensis*. The goal of the LIFE4FIR project (LIFE18/NAT/IT/000164, 2019–2023), is to improve the state of conservation of the natural population and to protect *A. nebrodensis* from extinction by responding to the main vulnerability factors involved. These are (1) the dramatic genetic erosion and fragmentation of the residual population; (2) the poor natural regeneration; (3) the risk of hybridization with possible non-native *Abies* species in neighboring plantations; (4) the shallow surface soil and rocky soil; (5) localized soil erosion; and (6) grazing by wild herbivores, especially fallow deer and wild boars.

To counteract these threats, the LIFE4FIR project is: (i) protecting the relict population (adult trees and natural regeneration); (ii) increasing the genetic diversity of the progeny and selection of outbred seedlings derived from exogamy; (iii) breeding selected outbred seedlings (iv) reforestation with 4000 selected seedlings to create re-diffusion nuclei of the species; (v) carrying out ex situ conservation by implementing a seed bank and cryobank for the long-term germplasm conservation. This article reports the main actions undertaken by the project and the results obtained after 3 years of activity.

2. Materials and Methods

2.1. A New Genetic Characterization of *A. nebrodensis*

Initially, the genetic relationships were investigated among the 30-*A. nebrodensis* adult trees and juvenile plants from the natural regeneration.

DNA was extracted from the needles and the genetic characterization was performed following a SNPs genotyping procedure using PCR-based OpenArrays technology (ThermoFisher Inc., Carlsbad, CA, USA). Based on previous genomic data on *A. nebrodensis*,

A. alba and *A. cephalonica* [19] a 120 SNP-array for the genotyping of *A. nebrodensis* individuals was developed.

Paternity tests were carried out on the 118 plantlets of the natural regeneration to determine the rate of outcrossing, inbreeding and self-fertilization and to assess the rate of introgression (i.e., possible hybridization) due to fertilization with pollen coming from the close plantations of non-native firs (*A. alba* and *A. cephalonica*). The same procedure was carried out on a sample of 2064 young plants raised in the local forest nursery 'Piano Noce'. This information was useful for their possible use in reforestation nuclei with increased genetic diversity.

2.2. Phytosanitary Surveys in the Nursery

Within the LIFE4FIR project, the nursery activity plays a fundamental role to produce healthy, vigorous, and genetically selected plants, for use in reforestation plots. The health status of the plants in the local 'Piano Noce' forest nursery was surveyed at the beginning of the project. The incidence of disorders, was recorded to control the impact of factors that can disturb the growth and vigor of the plants. Fungal isolations from twigs and needles affected by different disorders were also performed. The presence of *Phytophthora* spp. in the nursery was investigated in soil and roots samples collected from a pool of plants showing related symptoms of the pathogen such as chlorosis and defoliation.

2.3. Conservation of Genetic Purity of *Abies nebrodensis* and Improvement in Genetic Diversity

To control the risk of uncontrolled gene flow, a census and mapping were made on non-native fir trees present in reforested areas planted in the past in the vicinity of the *A. nebrodensis* population.

To enable exogamy between the isolated adult trees, controlled crosses were carried out. Manual pollination was performed according to information obtained from SNPs genotyping (see Section 2.1) on genetic distance between pairs of parents (co-ancestry), to obtain an offspring with a more variable genetic background. Hand pollinations were performed in May 2020 and 2022 by inserting mature male cones into terylene bags covering the opened female cones following a planned crossing combination scheme. The development of the cones inside the bags was monitored at 15-day intervals.

Seeds deriving from cross-pollinations were extracted separately in October from mature cones and sown at the forest nursery. To increase the germination rate of seeds, trials were conducted using different substrates and containers for the sowing. The seedlings obtained were then transplanted into bigger pots after one year.

2.4. Protection of the Relic Population by Monitoring the Health of the Trees, as Well as Fences and Video Surveillance System

In the natural habitat of *A. nebrodensis*, the harsh environmental conditions, the rocky soils and the out-of-control presence of herbivores such as wild boars and fallow deer together with the still active grazing of uncontrolled livestock, can considerably harm the natural regeneration and deteriorate the adult trees. In situ conservation strategies relies on a series of activities aimed at the preservation of *A. nebrodensis* by controlling their threats through (i) monitoring the phytosanitary conditions of the relic trees by visual inspection alongside multispectral imaging analysis using drone technology; (ii) monitoring growth of trees by registering the dendro-auxometric parameters; (iii) census and mapping of the natural regeneration; and (iv) extension and strengthening of the system of fences around each tree and the implementation of a video surveillance system.

2.5. Clonal Orchard and Reforestation with *A. nebrodensis* (Outbred 4000 Improved Seedlings) in 10 Plots in the Madonie Park in Areas Suitable for Reintroduction

A new clonal orchard has been planned, not only as a simple germplasm collection, but also to produce seed with increased genetic variability resulting from out-crossing. The aim is to broaden the genetic base of the population.

Reforestation trials are scheduled to be carried out in ten 2–3000 m² wide plots, with 4000 selected outbred seedlings.

2.6. Ex Situ Conservation Strategies

A procedure based on the use of X-rays was developed to quickly select in laboratory the full seeds, as the rate of empty seeds is usually high, thus lowering the significance of conservation efforts. Mature seeds were subjected to germination tests for their conservation at −18 °C (seed bank). In vitro germination was evaluated for excised embryos and pollen samples aimed at their conservation in the cryobank (at −196 °C). Trials were conducted to obtain embryogenic callus lines from excised embryos for cryopreservation.

3. Results

3.1. Genetic Analysis of the Natural Population and of Plants in the Nursery

The results obtained with Open-array technology clearly indicate that most of the adult trees are genetically close. The effective population size, which is a key parameter in population genetics to estimate the number of individuals that effectively contributes viable offspring to the next generation, is very low. More than 90% of seedlings growing at the natural populations originated from self-pollination. At the nursery, *A. nebrodensis* potted plants derived from open pollinated seed showed a self-fertilization rate similar to that found for the natural regeneration. Suspected hybrids coming from crosses with other firs were also found at the nursery.

3.2. Phytosanitary Inspection in the Nursery and in the Natural Population

More than 25,000 potted plants were counted in 2019 at the beginning of the project (Figure 3).



Figure 3. Potted plants of *A. nebrodensis* obtained from open pollinated seeds and raised in the local forest nursery Piano Noce.

The potted plants in the nursery were from 5 to 12 years old and derived from 12 mother trees of the natural population. The most frequently observed disorders were: chlorosis, 3.82%; reddened needles, 1.07%; stunted growth, 0.43%; defoliation, 0.17%; blighted shoots 0.1%; and small needles, 0.02%. Mortality was an average 2.4% in frequency, with a higher mortality rate (5%) among the 10-year-old potted plants which had experienced a single transplant. The disorders observed were scattered within the nursery. Isolations from soil samples and from the roots of the chlorotic and defoliated plants were negative for *Phytophthora* sp.

The fungal microflora isolated from reddened or yellowed needles and from blighted shoots were found to be represented by taxa that have a weak pathogenic activity and by saprophytes or endophytes. Ecophysiological analyses suggested that irrigation should be carried out at 5 days intervals when air temperatures exceed 25 °C.

3.3. Improvement of the Genetic Variability of the Offspring

In May 2020, controlled crosses were carried out on 24 trees. Crossings were performed according to 27 different combinations of parents and a total of 488 female cones were pollinated. Maturation of cones occurred regularly and bags were removed at the end of September to collect cones and extract seeds (Figure 4). To improve the germination success rate, planting procedure was optimized, including soil characteristics: the soil samples varied in salinity levels (EC) and total carbonate content, therefore a substrate for sowing was selected to increase the germination rate of seeds maintaining a pH of 5.5–6. More than 56,000 seeds were sown in December and April using QuickPot propagation trays (30 × 48.5 cm with 35 cells) positioning 5 seeds in each cell. After 5 months, a germination rate ranging from 10 to 80% among the combination of parents was achieved (Figure 5). The seeds obtained from the mother tree n. 19, both from controlled and open pollination, did not germinate at all. Currently more than 4000 outbred seedlings are growing in the nursery. In May 2022 controlled crosses were performed according to 23 cross-combinations. Overall, 121 bags were used to include 389 female cones.



Figure 4. Terylene bags isolating female cones for execution of controlled crosses in the mother trees no. 16 and 17.

3.4. Protection of the Relic Population by Monitoring the Health of the Trees, along with the Fences and Video Surveillance System

Twenty-six out of the 30 adult trees of the natural population of *A. nebrodensis* were found to show disorders and symptoms limited to a portion of the crown less than or equal to 15%. For 11 of these trees, damage appeared as a few little scattered blighted twigs and affected less than 1% of the foliage. Trees n. 12 and 18 showed disorders on a significant portion of the crown (between 16% and 50%), while tree n. 28 and 31 showed signs of a long-standing decline, with more than 50% of foliage damaged or missing.

The disorders most frequently observed were: reddened shoots and twigs; defoliation due to excessive shading; wounds due to herbivores; reduced leaves dimension and chlorosis; desiccation of twigs and branches (Figure 6a). These symptoms are often accompanied by wounds on the lower branches caused by wild herbivores (Figure 6b) or bark injuries due to the wind.



Figure 5. Full sib progenies (obtained from controlled crosses) of *A. nebrodensis* growing in propagation trays.



Figure 6. (a) Blight and necrosis of twigs and branches affecting the crown of the tree no. 12 of the *A. nebrodensis* population; (b) Damage due to feeding of wild herbivores in the lower part of the crown (mother tree no. 24).

The fungal taxa isolated from the needles affected by yellowing, reddening, partial or total necrosis were weak pathogens or endophytes. Nearly 80% of the fungi obtained were represented by 2 taxa: *Cytospora abietis* Sacc. (syn. *C. pinastri* Fr., anamorph of *Valsa abietis* (Fr.) Fr.) and *Rhizospora macrospora* Gourb. & M. Morelet.

On the basis of dendro-auxometric measurements, all the trees showed a moderate growth in trunk height, trunk and crown diameter compared to the surveys carried out during the previous LIFE project in 2000 (Table 1). Height of trees ranged from 14.4 m (tree n. 2) to 0.45 m (tree n. 28). Trunk diameter at 1.3 m from the ground ranged between 49.9 cm (tree n. 13) to 2 cm (tree n. 32). Trunk diameter of plants n. 4, 28 and 31 is referred to the base since their height was less than 1.3 m. Fifteen out of 30 trees were in good vegetative conditions and have a balanced shape of the crown. Ten trees showed less good vegetative conditions compared to the previous ones, due to the poor edaphic conditions or exposition to the prevailing winds. These trees are characterized by a crown which is less regular in shape, sometimes lacking in some portions. Trees n. 9, 28 and 31 were suffering from stunted growth due to the harsh conditions at the site and damage caused by herbivores.

Table 1. Dendro-auxometric measurements of trees of the relic population of *A. nebrodensis*.

Plant n.	Trunk Height (m)		Trunk Diameter at 1.3 m (cm)		Crown Diameter (m)	
	2000	2022	2000	2022	2000	2022
1	7.4	6.5	30	41.3	7.3	7.6
2	(N) 10.2; (S) 12.2	(N) 10.7; (S) 14.4	(N) 31.9; (S) 41.4	42.9	8.8	9.2
4	1.1	0.85	10.6 *	10.5 *	3.2	3.2
6	6.2	7.8	(W) 24.2; (E) 21	23.8	7.5	7.5
7	(W) 2.58; (E) 5.15	(W) 3.4; (E) 5.7	(W) 15.9; (E) 8.9	21.3	4.6	5.4
8	9	11	32	43	6.1	7
9	2.9	1.8	13	15.9	3	3.6
10	6.4	(W) 7.5; (E) 4	(W) 38; (E) 12.5	(W) 40.7; (E) 11	6.5	7.4
11	(S–W) 6.8	8.2	(N) 25; (S) 18.4	(N) 28.3; (S) 19	5.4	5.4
12	8.2	8.5	29.3	35	6	7.6
13	9.5	11.1	37.6	49.9	7.5	9.3
14	4.2	7.2	17.1	29.6	5.7	6.7
15	5.9	8.5	16.8	23.5	4.4	5.5
16	4.1	5.3	24.8	34	5.4	6.3
17	9.5	10.7	45.8	50.2	7.1	8
18	7	7.6	22.3	25.4	4.25	4.8
19	4.1	5.5	12.7	17.8	3.6	4.75
20	8.5	9.2	19.1	21.9	3.4	3.4
21	10.6	11.6	35	40.7	6.6	8
22	10.2	12	33.12	38.1	5.5	5.9
23	6.3	7.5	(N) 23; (S) 22	(N) 24.8; (S) 26	4.5	4.5
24	2.9	3.1	6	12.4	3.2	3.9
25	2.9	3.3	5	12.7	3.2	3.5
26	3.9	6	8.3	11.4	2.4	3.7
27	7.2	9.5	(W) 30.6; (E) 26.35	(W) 36; (S) 35	5.4	7
28	0.4	0.4	2 *	14.9 *	1.7	1.7
29	8.8	10.5	19.1	23.8	5.7	5.7
30	0.6	1.7	2	2.2	0.8	1.6
31	1	1.25	4 *	4.77 *	0.95	1.3
32		1.96		2		1.8

Values of trunk height, trunk and crown diameter of the 30 individuals of *A. nebrodensis* in the natural population. Comparison between data obtained during the course of LIFE4FIR project (2022) and the previous LIFE project [16] ended in 2004 are reported. (*): measurement of the trunk diameter at the base are shown for plants which height was less than 1.3 m.

A census carried out in summer and fall 2020 on the natural regeneration allowed the detection of 484 juveniles of *A. nebrodensis*, subdivided among 15 mother trees. Maps were obtained reporting the distance and the azimuth angle of each detected accession for each mother tree (Figure 7). Mother tree n. 10 showed the highest number of plants of the natural regeneration among the *A. nebrodensis* relic trees with 169 accessions recorded. The age of the young plants ranged from 6 months to 24–26 years (Figure 8). Many young plants of the natural regeneration were located outside the old fences.

A series of maps was obtained by drone surveys: 3D reshaped DTM showing the distribution of the trees in relation to morphology of the land; infrared maps showed the reflectance of the cover canopy of the tree based on their water content and chlorophyll absorption; NDVI maps reported the greenness and health status of the habitat vegetation highlighting where vegetation is thriving and where it is under stress.



Figure 7. Young plants of the natural regeneration of *A. nebrodensis* growing on the beech litter.

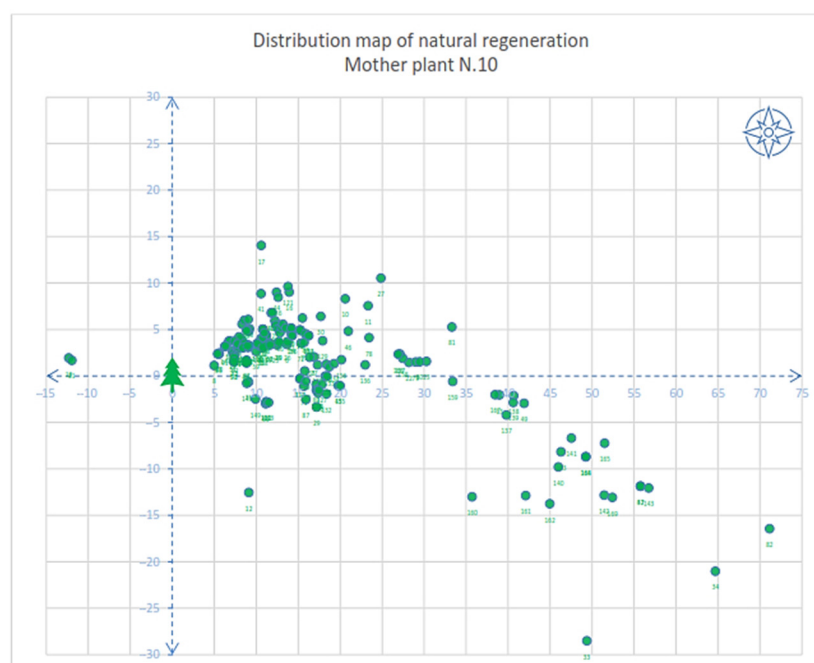


Figure 8. The distribution maps of the natural regeneration were elaborated for each mother tree based on the azimuth coordinates and the distance measured for each accession referred to its purported mother tree. Both axes report linear metric measurements. As example, here is reported the map obtained for the mother tree n. 10, the one with the most abundant natural regeneration around.

The new fences are made of chestnut poles with a top diameter of no less than 7 cm and a length of no less than 2.40 m (Figure 9). Before installation, the bottom 60 cm was caulked. They were then placed 2 m away from each and at a depth of 40 cm in the ground. The metal net is made of 1.60 m height galvanized iron wire with a degrading mesh, with a minimum weight of 0.70 kg per linear meter. Each fence has been equipped with a 1.5 m wide gate entrance. Overall, the fenced area around the trees has been increased from 1420 m² to 2144 m² and perimeter of fences installed around the trees ranges from 40 m to 230 m. The new fences were installed to include and protect the young plants of the natural regeneration detected in the last census.



Figure 9. The 2 m tall new fence installed around the *A. nebrodensis* tree n. 7.

3.5. Clonal Orchard and Reforestation with *A. nebrodensis* (Outbred 4000 Improved Seedlings) in 10 Plots in the Madonie Park in Areas Suitable for the Reintroduction

Graftings were carried out in March–April 2022 for the vegetative propagation of the *A. nebrodensis* trees of the natural population, following the veneer-side procedure: 23 out of 30 *A. nebrodensis* trees were grafted, and 640 grafted plants were obtained. In May 2022 the rate of successful grafts was higher than 50%.

Ten sites, varying from 800 to 1600 m a.s.l., were selected for reforestation in the Madonie Regional Park. The sites contained the following climax associations: *Quercion ilicis*, *Quercion roboris*, and *Geranio versicoloris*-*Fagion*. Some plots were selected in areas already covered by forest trees to benefit from the protection of extant plants. The soils are of quartz sandstone type, the same type of substrate where the natural population grows, and of calcareous type. Most of the plots are northern facing, which is the exposure that the species naturally prefers. All plots will be protected by 2 m high fences before planting will start. The production of seedlings to be used in the reforestation program is ongoing. Currently, more than 4000 seedlings derived from controlled crosses carried out in 2020 are being raised in the Piano Noce nursery (see Section 3.3). Further seedlings are expected from the controlled crosses of 2022. Studies on mycorrhizal fungi have been developed at University of Palermo where an isolate of *Pisolithus arhizus*, a Basidiomycete fungus well represented in the forest soils of the Madonie on various conifers, has been successfully tested for mycorrhization of selected seedlings.

Planting will take place in the last year of the project (2023) depending on the growth of seedlings in the nursery.

3.6. Ex Situ Conservation Strategies

A protocol for conservation of seeds at low temperature ($-18\text{ }^{\circ}\text{C}$) has been developed (Figure 10b–d) following the international standard for long-term seed conservation (FAO/IPGRI 1994) (Figure 10). Sample seed from 11 mother trees were prepared for permanent storage in the seed-bank, inside labeled jars (Figure 11a). Their moisture content ranged from 5.56 to 8.18%. For conservation in liquid nitrogen at $-196\text{ }^{\circ}\text{C}$ seeds were inserted in cryovials. Cryopreserved seeds showed a viability of 85–90% (Figure 11b) while excised zygotic embryos showed a germination rate of 90–95% after cryopreservation. Pollen samples collected in 2020 and 2022 from 23 fertile trees were tested for cryopreservation before pollen immersion in liquid nitrogen. The *in vitro* germination, viability (TTC test) and the moisture content (about 8%) were evaluated. Viability after cryopreservation ranged from 88 to 96% without significant deviation from fresh pollen. *In vitro* embryo-

genic callus lines have been developed, but the procedure for obtaining somatic embryos has yet to be fine-tuned. The seed bank and the cryobank have been established, in a dedicated room, at the MAN (Museum of *Abies nebrodensis*) in Polizzi Generosa.

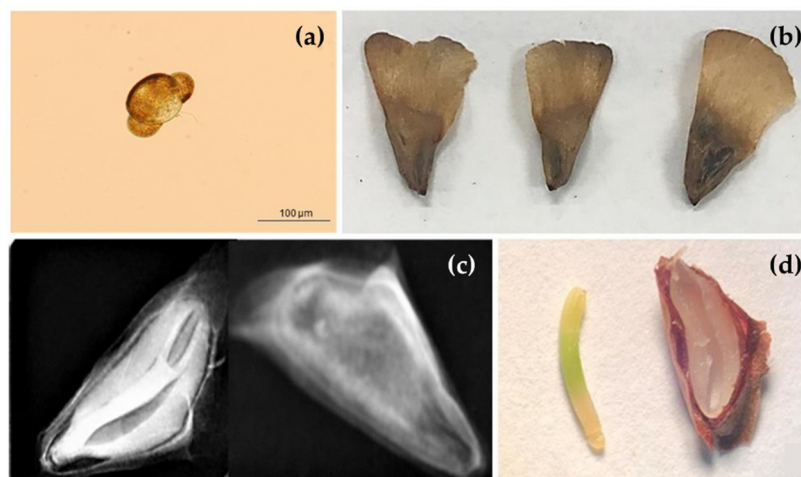


Figure 10. *Abies nebrodensis*: (a) a grain pollen (100×); (b) seeds after extraction and cleaning; (c) seeds at the X-ray device: a full seed (left); an empty seed (right); (d) an excised embryo from mature seed.



Figure 11. Long-term conservation of *A. nebrodensis* by (a) Seed bank ($-18\text{ }^{\circ}\text{C}$) for seeds maintenance and (b) Cryobank ($-196\text{ }^{\circ}\text{C}$) for seed, pollen, embryos and embryogenic callus lines.

4. Discussion

The Madonie fir, is a critically endangered species, and is a clear example of the consequences of the unsustainable exploitation of natural resources, leading to habitat loss and fragmentation as well as genetic erosion. The LIFE4FIR project was set up to respond to the key vulnerability issues affecting the *A. nebrodensis* population. Although the concrete effects of most actions will be visible in the next years or decades, a solid basis of good practices is being developed to be continued after the project ends to improve the conservation status of the species and reduce its risk of extinction.

There are many studies on the importance of ex situ and in situ conservation strategies of various forest species, particularly conifers, whose habitat is impacted by drought, fires, flooding as well as by human pressure [20–23].

Protecting *A. nebrodensis* from potential extinction represents a good opportunity to raise awareness on the role of biodiversity in ecosystems and the risks associated with

its loss, and on ability of balanced ecosystems to provide the community with a series of goods, resources and services and to cope with the pressures of climate change.

Unlike previous studies, the analysis of the genetic structure of the population conducted within the LIFE4FIR project, revealed a high rate of relatedness among the adult trees and a high rate of self-fertilization among the young plants of the natural regeneration [6,9–11,17]. The natural population of *A. nebrodensis* suffers from a significant level of inbreeding, which could have a strong negative impact on the evolutionary dynamics of the species. Therefore, to increase genetic variability of the population, it is essential to carry out outcrossing between trees genetically more distant and, if possible, with low levels of inbreeding and homozygosity. In fact, genetic rescue can maintain evolutionary potential and, in the present case, prevent the high extinction risk of *A. nebrodensis*.

The need to create new outbred progenies through controlled crosses also emerged from the high rate of self-fertilization that resulted from the genetic analyses carried out on young plants raised in the local nursery. This data in fact suggested that plants grown in the nursery before the start of LIFE4FIR might not be suitable for establishing the new reforestation nuclei nor for increasing the genetic diversity of the species. The microarrays SNPs genotyping and paternity tests performed during this project led to the definition of 30 recommended crosses based on co-ancestry of parents to increase the genetic variability of the progeny as much as possible. At an ecological and biological level, genotyping and paternity tests are the basis for creating new genetic pools and increasing the genetic diversity of the species.

Safeguarding the genetic integrity *A. nebrodensis* is of primary importance, especially as genetic analyses of the natural regeneration and of seedlings raised in the local forest nursery revealed suspected hybrids with other *Abies* species. Since plantations of *A. cephalonica* and *A. alba* that had been previously established are in the vicinity of the natural area of *A. nebrodensis*, surveys were carried out on the risk that they represent for maintaining the genetic purity of *A. nebrodensis*. In fact, *A. cephalonica* found very favourable conditions for growing and regeneration after its introduction in the Madonie, leading the species to be considered naturalized in Sicily and representing a real threat for the genetic integrity of *A. nebrodensis*, since the closest trees are 3 and 4.8 km far from the natural population. Therefore, the census and mapping of non-native fir plantations carried out in the LIFE4FIR project are key to planning interventions aimed at removal of *A. cephalonica* plantations.

Controlled crosses made in 2020 gave good results thanks to the abundant fruiting of *A. nebrodensis* in that year. Unfortunately, the extremely variable flowering from one year to the next is a major obstacle to planning this activity. In fact, in 2021 the fruiting was very poor and controlled crosses could not be carried out, while in 2022 the activity resumed with a good number of female cones that were subjected to hand pollination. These are expected to produce many other outbred seedlings.

The phytosanitary surveys carried out in the local nursery showed overall a better picture than expected. Incidence of the observed disorders and mortality were substantially contained. Surveys in the nursery highlighted the need to improve some measures in raising plants, such as the standardized preparation of the soil mixture for filling the containers, the need for regular and more frequent transplants, watering every five days when the temperature exceeds 25 °C, adequate shading.

The surveys carried out excluded the presence of aggressive necrotrophic pathogens, such as some species of oomycetes (e.g., *Phytophthora* spp.), that can seriously jeopardize sowing and nursery production due to the favourable conditions that these species can find in this place—frequent irrigation, plants close one another, infected soils batch etc. [24–27]. Appropriate cultivation practices are key to ensuring the best growing conditions for the seedlings produced and selected during the project, and to providing healthy and vigorous planting stock for reforestation.

Despite the harsh environment where the relic trees of the *A. nebrodensis* population live, the health status of the natural population was not particularly poor. Most of the trees surveyed (26 out of 30) showed disorders on a relatively small portion of the crown,

not exceeding 15%. Only the mother trees 28 and 31 showed a stunted growth and an advanced decline, mainly due to the harsh site conditions and the destructive action of wild herbivores. Most disorders are due to environmental stress, such as summer drought, late frosts, high temperatures reached by the bare rocks during summer, and the intense solar radiation, in addition to the damage caused by boars and deer. All fungi that were isolated were classified as weak pathogens, endophytes or saprophytes, whose development is associated with the environmental disturbances reported above.

Measuring the dendro-auxometric parameters of each tree is useful for monitoring growth and vigour over time, thereby produced a baseline to refer to in the future to assess evolution of the trees in quantitative terms, also in relation to climatic fluctuations and to the protection measures carried out in the course of the project.

The shape and growth of the crowns mainly depends on the edaphic conditions of the site, along with water availability and exposure to strong winds. Trees located within beech groves can exploit the deeper and fertile soil, and are characterized by a higher growth rate than the others. The trees with poor growth tend to be located on rocky ridges, adjacent to stony ground with almost no soil and are exposed to the prevailing winds. Unexpectedly, some trees growing in the middle of screes appear to have a very good crown shape and health. Overall the trees reflect a substantial long-standing equilibrium with the surrounding environment, although they exhibit the signs of living at the upper limits of their natural range.

The in situ measures are aimed at managing the conservation of the natural population in a sustainable way, based on the needs of the species at this stage and on the main threats. The new fences have reduced the impact of wild herbivores, above all fallow deer and wild boars, and the grazing of uncontrolled livestock on the natural regeneration and the adult trees. This should promote the settlement of the natural regeneration which has already resulted in a gradual increase compared to the previous survey carried out in 2014 (484 trees in 2020 vs 274 in 2014). Protection against the unsustainable numbers of wild herbivores has been supported by the management plan for the fallow deer population in the Madonie Regional Park for 2021–2025 authorized by the Regional Agriculture Department of Sicily. A monitoring and control plan has been launched for wild boars with captures and culling planned through movable fences also in Area A of integral nature reserve where the *A. nebrodensis* population is located.

The video surveillance system enables operators to monitor grazing and the anthropogenic pressure in the sites most frequented by visitors and should deter possible environmental crimes.

Various reforestation projects have been implemented in the last 40 years, also within the framework of the previous LIFE00 NAT/IT/007228 project using seedlings obtained from open pollinated seed produced by trees from the natural population [16]. Plantations were built in potentially suitable stations of the Park at various distances from the natural *A. nebrodensis* population.

Recent surveys have shown that the best results were obtained in parcels with a quartz sandstone substrate, facing north, at 850 to 1600 m a.s.l., with the presence of mesophilic deciduous, holm oak (*Quercus ilex* L.) and acidophilic beech (*Fagus sylvatica* L.) woods. The average survival rate in these sites was 62% [28].

The current LIFE4FIR project will consider results from previous reforestation trials to choose the most suitable sites for *A. nebrodensis* growth. Compared to the plantations carried out in the past, the new reforestation plots will be made using selected outbred seedlings obtained from crosses among the mature extant genotypes. This hopefully should lead to an increase in genetic diversity, and should stop the genetic erosion and reduce homozygosity while facilitating the adaptation capacity of the species in relation to environmental stresses and to climate change. Furthermore, the re-diffusion cores will be properly realized in areas where *A. nebrodensis* can eventually have the chance to migrate upward as a form of resilience to global warming. Population dynamism of *A. nebrodensis* can be re-started if a

proper strategy is established to improve the gene pool and promote the future dynamism of the species in relation to different biotic and abiotic pressures.

The LIFE4FIR project has set up a germplasm bank, as a seed-bank and a cryobank, for storing tissue and organs of *A. nebrodensis*, in order to create a safe long-term ex situ repository for this endangered species. In addition to traditional seed-banking and clonal orchards for the ex situ conservation of tree genetic resources, cryopreservation is rapidly evolving [29–33]. It is a complementary aid in the plant biodiversity conservation programs, providing a further guarantee against accidental loss of genetic resources.

After the development of protocols, the seedbank and the cryobank were set up at the Museum of *Abies nebrodensis* (MAN) in Polizzi Generosa. This museum was chosen as it is frequently visited by scientists, schools, study groups and the general public. The germplasm bank is thus being used to disseminate knowledge on current strategies for conserving threatened genetic resources.

In summary, the key strengths of this project so far have been: (1) good growth conditions of the seedlings in the nursery; (2) implementation of a new fence system; (3) the natural regeneration is expanding; (4) stable health conditions of trees of the relic population; (5) more than 4000 seedlings obtained from controlled crosses in 2020 are now growing in the nursery; and (6) the a seed bank and cryobank have been set up.

However, there have also been some limitations: (1) the high rate of inbreeding and self-fertilization in the natural population; (2) the nursery plants bred in the nursery before the start of the LIFE4FIR project were largely self-fertilized or hybrids and cannot be used for reforestation; (3) the extremely variable flowering detected in this forest species; (4) the populations of fallow deer and wild boar have reached unsustainable levels.

LIFE4FIR has implemented a strategy to respond to the key vulnerability factors affecting the *A. nebrodensis* population. Although the real effects will only be visible in the next few years or decades, efforts are aimed at developing a solid bases of good practices to be sustained even after the end of the project. The key elements of this strategy, which we believe should be adhered to in the future both in this and similar projects, entail the regular monitoring/maintenance of: (1) the new fences; (2) the cameras and solar panels; (3) the health status of trees of the natural population and natural regeneration. In addition, (4) post-planting treatments in the reforestation plots is needed; (5) the established clonal orchard needs care and regular attention; (6) the; nursery production of improved *A. nebrodensis* should be continued following the indications emerged; (7) the cryobank in the museum needs to be maintained by refilling the liquid nitrogen; and (8) periodical assays of vitality and germination tests are needed, in order to determine when sampling should be renewed.

We believe that the conservation strategy developed and implemented in the Life4fir project for *A. nebrodensis* can represent a reference to be replicated for the protection of other endangered circum-Mediterranean firs. These include *A. cilicica* in Lebanon and Syria; *A. nordmanniana* subsp. *equi-trojani* in Turkey; *A. pinsapo* in Spain and Morocco (var. *marocana*). The main threats affecting these species in turn are fire and grazing, isolations of populations and fragmentation [34], urban pressure, hybridization. This kind of threats are common to some other Mediterranean conifers, like *Pinus leucodermis* (Italy), *Juniperus thurifera* and *Tetraclinis articulata* in Spain and Malta.

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