LIFE4FIR – Project LIFE18 NAT/IT/000164

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

Report on the Evaluation of genetic diversity of adult plants and natural regeneration
A1.1

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

Parco delle Madonie

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A1.1 Evaluation of genetic diversity of adult plants and natural regeneration.

1. Introduction. General aims of action A1.1

The general aim of this action is the evaluation of the genetic variability and the genetic relationships among the 30-adult trees and the 118 juvenile plants from the natural population of *Abies nebrodensis*. SNPs genotyping was used to assess the genotype of these individuals. In particular, we used the OpenArrays technology for the genetic characterization of both the adult trees and juveniles from the natural population. We studied the genetic diversity and structure of the natural population of *A. nebrodensis*. Then, paternity tests were carried out on the seedlings to determine the rate of outcrossing (cross between unrelated individuals), inbreeding and self-fertilization and to assess the rate of introgression (eventual hybridization) due to fertilization of female cones with pollen coming from alien firs (*Abies alba* and *Abies cephalonica*). All the followed steps to achieve the aims of this actions are detailed in the following sections of this report.

2. Adequacy and availability of SNPs genotyping techniques

Originally, it was intended to use GoldenGate Genotyping with VeraCode technology (Illumina Inc., United States). However, this molecular assay is currently in disuse and we did not find any research center or biotechnology company to perform these analyses. Alternatively, we used the PCR-based OpenArrays technology (Thermofisher Inc., United States), which provides a robust and flexible platform for SNP genotyping and provides superior data quality and high sample throughput at low per-sample costs, making it ideally suited for studies involving large volumes of samples. Based on previous genomic data (Balao *et al.* 2020, Ann Bot 125: 495-507), we developed a 120 SNP-array for genotyping of *A. nebrodensis* individuals 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. A PCoA (Principal coordinates analysis) conducted at the individual level revealed that the first two factors explained 44.3% and 20.3% of the total variation, respectively, and mostly separated the individuals of *A. nebrodensis* from those of *A. alba* and *A. cephalonica* (Figure 1). The remaining 100 SNPs were selected for the paternity test for *A. nebrodensis* based on their high diversity.

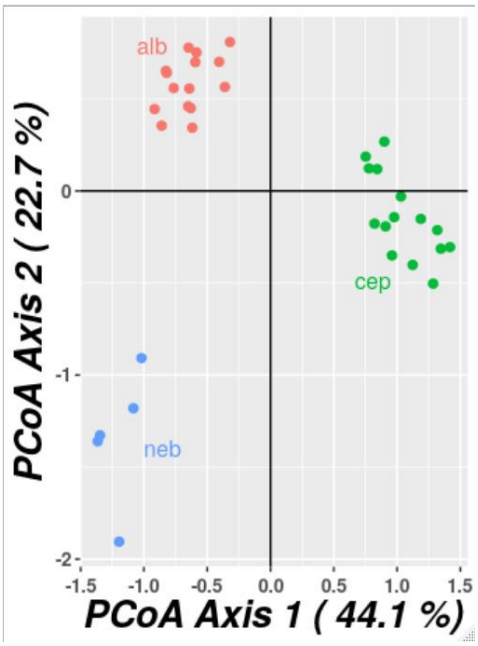

Figure 1. Ordinations of 35 individuals of *A. nebrodensis* (neb; blue), *A. alba* (alb; red) and *A. cephalonica* (cep; green) species based on PCoA. The first two factors explained 44.3% and 20.3% of the total variation, respectively.

3. Laboratory equipment and consumables used

In view of the large volume of samples to be analyzed in action A.1.1 and A.1.2, we concluded the use of a DNA extraction protocol based on 96-well plate format was the option that best suited our needs. The NucleoMag Plant (Macherey-Nagel Inc., Germany) was the DNA extraction kit selected for preparation of DNA from plant samples. The DNA extraction of this procedure is based on reversible adsorption of nucleic acids to paramagnetic beads under appropriate buffer conditions, obtaining high quality DNA that can directly be used for downstream applications. We acquired specific laboratory equipment for isolation of DNA using this extraction protocol based on 96-well plate format when necessary.

4. Sampling and DNA extractions

In November 2019, all the mature trees (n=30) and young individuals over 5-year old (n=118) in the natural population of *A. nebrodensis* were sampled. All these samples were preserved in silica gel until the subsequent DNA extractions.

We carried out multiple tests to optimize the DNA extractions using the NucleoMag Plant kit in *A. nebrodensis* samples, obtaining as a consequence satisfactory DNA concentration. We followed the manufacturer protocol with the following modifications to optimize DNA extractions:

- The volume of the MC1 elution buffer was increased to 650 **µ**L to ensure the transfer of 400 **µ**L of clear lysate after centrifugation (step 2).
- Samples were centrifuged at lower speed than recommended because of centrifuge limitations. However, we increased the centrifugation time to 40 minutes (double than recommended), obtaining clear lysates for all samples.
- Waiting time in each wash step was extended to 5 minutes instead of 2 minutes to ensure the separation of the magnetic beads against the side of the wells when placing the Squarewell Block on the NucleoMag SEP magnetic separator.
- RAW buffer was used instead of the wash MC4 buffer to get cleaner DNA.
- The wash step using the MC5 buffer was omitted. This step of the protocol is to remove traces of ethanol, but we observed than frequently affected the quantity of DNA concentrations. Instead, samples were kept at room temperature for 10 minutes to ensure that traces of ethanol were volatilized.

From January to September 2020 - this period covers a 3-month halt in the activity due to national emergency caused by COVID-19 – all DNA extractions were performed. DNA extractions were repeated for those samples of which we obtained insufficient DNA concentrations. An 86.3% of success (considering as such a cutoff of 10 ng/**µ**L DNA concentration) was reached. The average concentration of the 30-adult trees and the 118 juvenile plants from the natural regeneration was 51.41 ± 1.64 ng/**µ**L (mean ± SE).

5. Assessment of the validity of the OpenArrays technology in *A. nebrodensis*

The validity of the OpenArrays technology for SNPs genotyping of *A. nebrodensis* was tested for the first time by genotyping samples in duplicate. In particular, 12 sibling seedlings from the same mother with different DNA concentrations (ranging from 15.3 to 77.8 ng/**µ**L) were selected. We obtained a replicability higher than 99% when comparing the results obtained

for each duplicate, ensuring the validity of the genotyping data obtained. The proportion of genotypes (i.e. call rates) obtained for all individuals was similar, independently of the starting DNA concentration of samples. This increased the final number of samples to be genotyped, given that even samples with medium-low or low DNA concentrations can be used for the genetic characterization. Genotyping failures were detected in 64 loci, but only eight of them had a percentage of failure higher than 50% (Figure 2A). Please note that a small portion of loci with a high percentage of failure is always inherent to any genotyping technique.

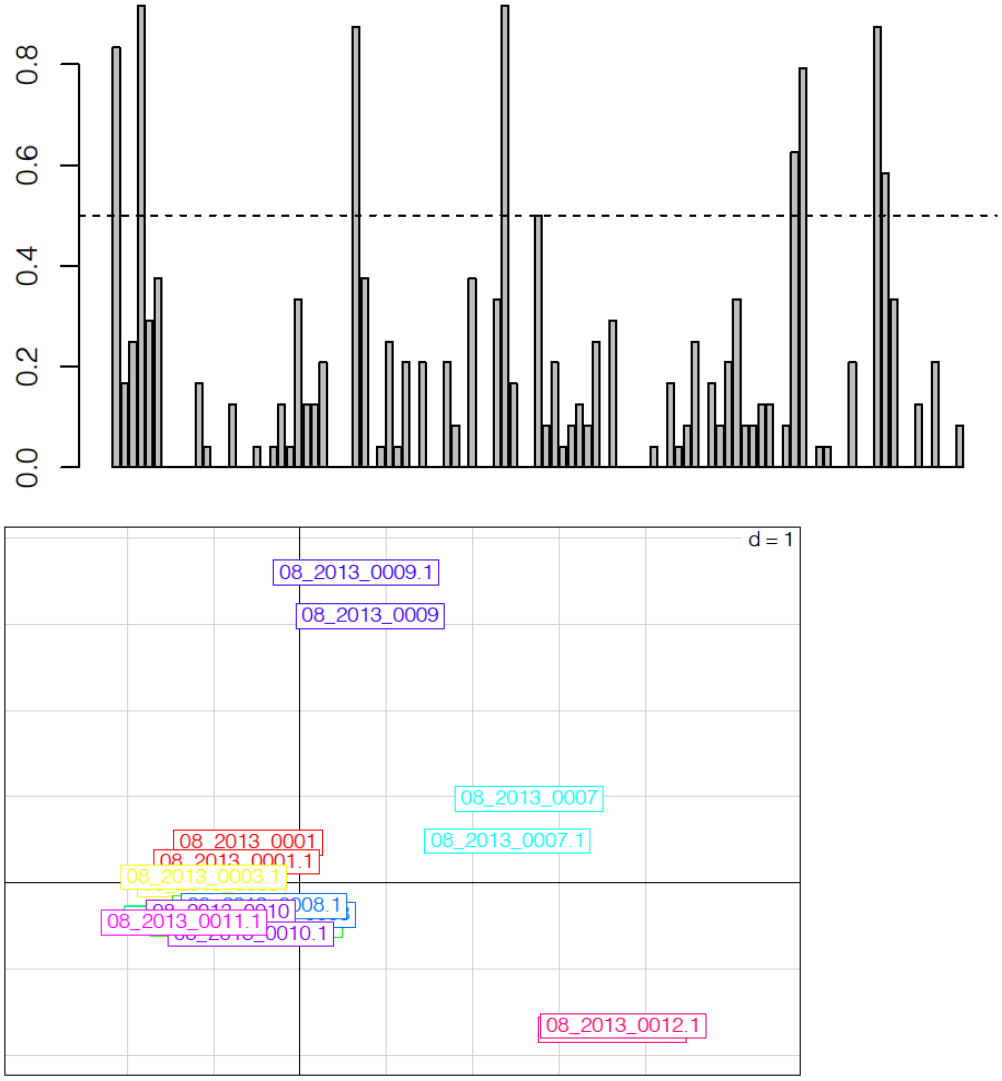

Figure 2. (A) Percentage of failure (Y-axis) for each of the 120 SNPs (X-axis). The dashed line represents a cutoff of 50% of failure. (B) Two-dimensional scatterplot extracted from the Principal Component Analysis (PCA) of the 12 individuals genotyped in duplicate. Each individual is represented with different colors. Individual names correspond to labels assigned during the sampling (from 08_2013_0001 to 08_2013_0012). Note that sample names ended in ".1" correspond to duplicates.

In addition, preliminary Principal Component Analysis (PCA) revealed genetic differentiation among samples, even though they share the same mother, and showed that duplicates clustered together (Figure 2B). **According to these results, we concluded that the used markers allow the correct assignment of progeny from the same mother and can help to elucidate the genetic relatedness among the adult trees.**

6. Analysis of the genetic diversity and structure of the natural population of *A. nebrodensis*

The 30-adult trees of *A. nebrodensis* were genotyped up to three times (four to six times for challenging samples) to try to recover the 120 informative SNPs for all individuals. After trimming those loci with a high percentage of missing data, we recovered high quality genetic information from 100 loci sites (3.12% of missing data). We used this data to study population structuring, that is if the genetic structure of the population could be subdivided. We used the Discriminant Analysis of Principal Components (DAPC), performed by using the "*adegenet*" package for the R software v.4.0.3, to study the population subdivision in *A. nebrodensis*. We conclusively found the existence of only three genetic clusters within this population (by K-mean clustering; Figure 3A), which is reasonable due to the high levels of selfing and inbreeding and outcrosses occur between related individuals (not random mating), creating genetic substructures of the population. When we represented the genotypes of each individual in a genetic network, we found a high interconnectivity of the three genetic clusters. It should be noted that individuals 21M and 31M appeared outliers of the network (i.e. with very low interconnectivity with the rest of individuals). Below, we discuss the genetic peculiarity of these plants.

Additionally, we analyzed the natural population of *A. nebrodensis* using the COLONY software v.2.0.6.6. Based on the estimated pedigree, we assessed the inbreeding level of the population by estimating the inbreeding coefficient (F_{is}) of the population. For *A. nebrodensis*, F_{is} = 0.3571 when inbreeding was accounted in the analysis, obtaining a higher value (F_{is} = 0.5263) when selfing was considered in the analysis. These F_{is} values point to a remarkable level of inbreeding for *A. nebrodensis* individuals. Furthermore, **we estimated the effective population size (N_e), which is a key parameter in population genetics that estimate the number of individuals that effectively contributes offspring to the next generation. We found that N_e = 6 (3 – 21; Confidence Interval 95%) for the *A. nebrodensis* population**, reflecting the strong impact of genetic drift and inbreeding on the

evolutionary dynamics of this population. Lastly, we analyzed the genetic diversity of adult trees by calculating the following coefficients of heterozygosity, homozygosity and inbreeding at the individual level: PH_t (proportion of heterozygous loci in an individual), H_s (Standardized heterozygosity based on the mean expected heterozygosity), *IR* (internal relatedness), *HL* (homozygosity by locus) and *INBR* (inbreeding coefficient). These coefficients mainly reflected the moderate homozygosity of *A. nebrodensis* plants due to inbreeding and selfing.

Finally, we calculated the Ritland estimator (*RIT*) of pairwise co-ancestry between adult individuals to know the genetic relatedness of the 30-adult trees of *A. nebrodensis*. Pairwise comparisons of the individuals genetically more distant will result in negative *RIT* values, whereas positive values will be obtained for comparisons of individuals genetically more similar. For clarity, we represented the pairwise co-ancestry of adult trees in Figure 4 (original values are showed in Supplementary Table 1). **For regeneration activities in the population, outcrossing of individuals with more negative *RIT* values (i.e. larger red circles in Figure 4) are desirable when possible**. A list of the 30 most recommended crosses between mature trees of *A. nebrodensis* can be found in Table 2. Unfortunately, we do not have concise information about the reproductive maturation of adult individuals of this population, so please note some of the recommended outcrossing may involve non-reproductive individuals.

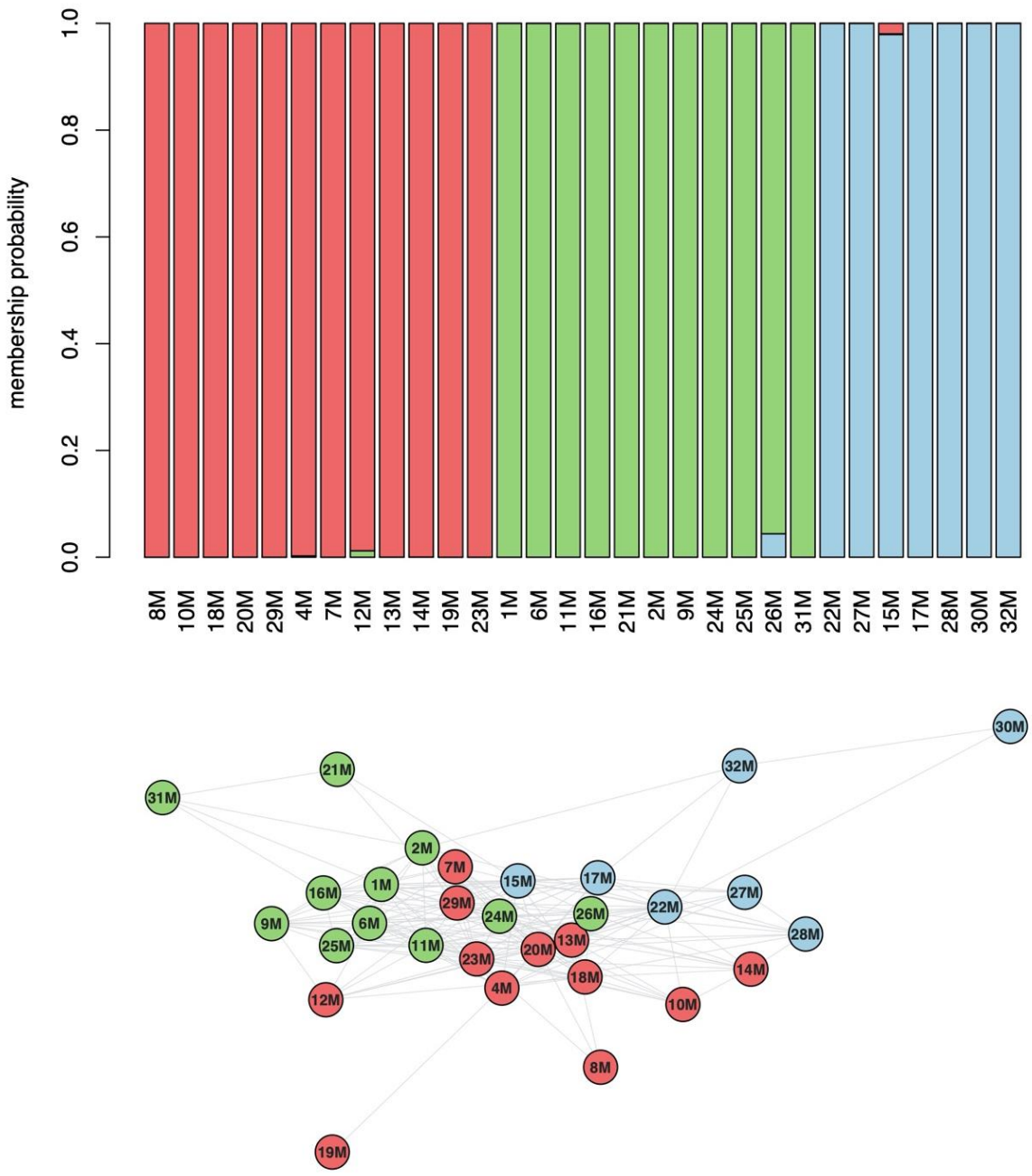

Figure 3. (A) Barplot based on a discriminant analysis of principal components (DAPC) of the genetic information from 100 loci, in which the assignment probability to each of the three genetic clusters (red, green and blue) of the 30-adult trees of *A. nebrodensis* is represented. (B) Genetic network of the 30 genotypes of the *A. nebrodensis* population. Genotypes were colored with red, green and blue colors according to their assignment to each of the three genetic clusters of the population.

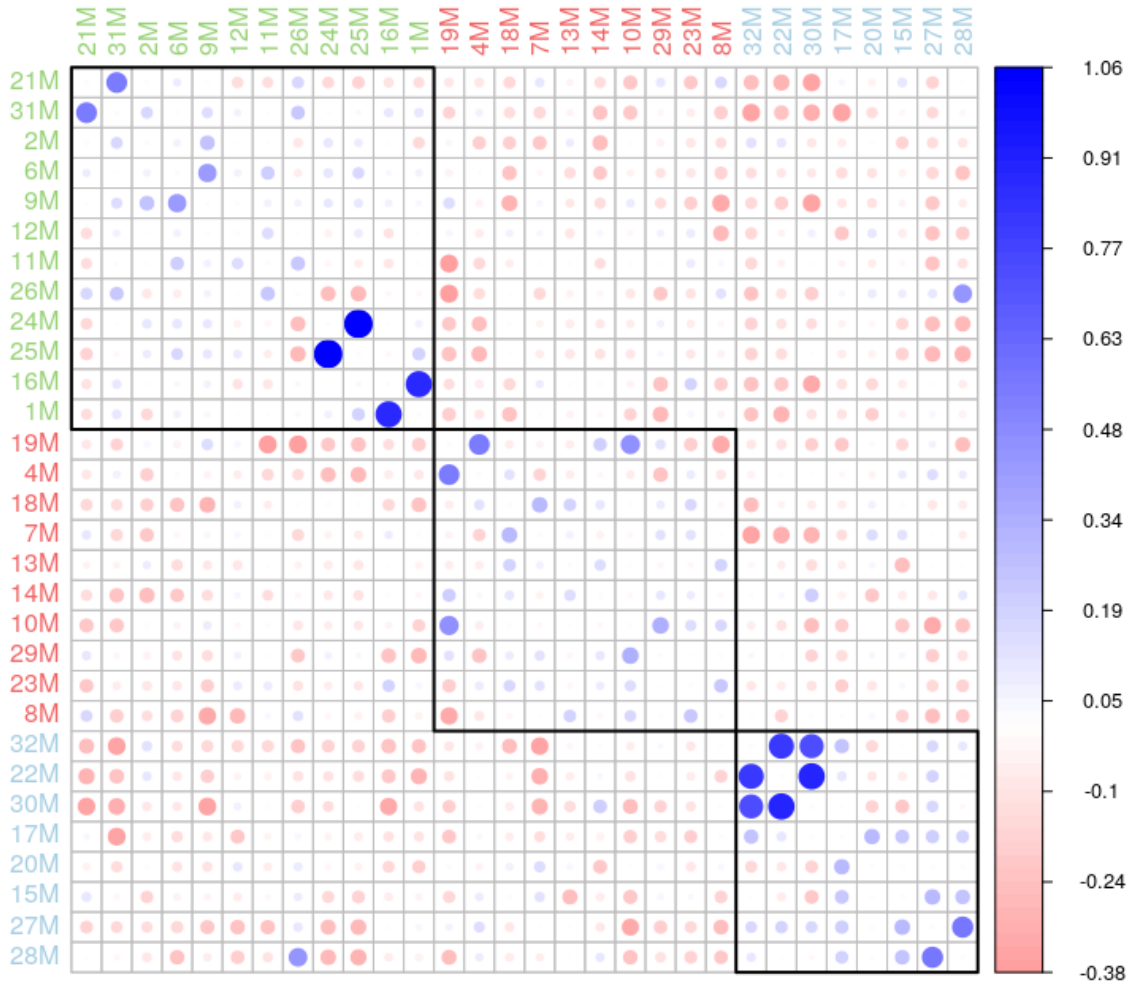

Figure 4. Matrix of pairwise co-ancestry between the 30-adult of *A. nebrodensis*. *RIT* pairwise estimations are represented by means of relative point size and colors represent either positive (blue) or negative (red) values. Font color (green, red and blue) corresponds to each of the three genetic clusters previously described. Pairwise comparisons within the same genetic cluster are framed within a box.

7. Summary

The natural population of *A. nebrodensis* suffers a significant level of inbreeding, which could have a strong negative impact on the evolutionary dynamics of this population. In our opinion, spontaneous mutations will not be enough to increase genetic variability of the population, so active management of this population is necessary. It is essential to carry out future outcrossing using individuals genetically more distant and, if possible, with low levels of inbreeding and homozygosity. The possibility of genetic rescue should be considered as a way to maintain evolutionary potential and to prevent the high extinction risk of *A. nebrodensis* population.

7. Paternity test of natural regeneration plants of *A. nebrodensis*

Paternity tests were carried out on natural regeneration seedlings/saplings to determine the rate of outcrossing, inbreeding and self-fertilization and to assess the eventual hybridization due to pollen coming from non-native *Abies* species. Paternity tests were performed using the COLONY software v. 2.0.6.6. We inferred the origin (considering as such the identification of one or both parents) of all young individuals except for three (21.3.P, 21.4.P and 21.5.P). Ninety-one plants from natural regeneration (77.1%) were found to be assigned to their putative mother plants (according the codes appearing in the labels of these individuals), whereas 24 seedlings were assigned to a different mother than appearing in their labels (see Table 3 for further details). Many of these 24 seedlings were assigned to mother plants that were geographically close (e.g. we discovered several seedlings that were offspring from plant 10M but were labeled as offspring of plant 11M; both trees are separated by a few meters). Only in six cases (11.10.P, 11.11.P, 11.12.P, 11.13.P, 11.18.P and 11.19.P) seedlings were found to be assigned to geographically distant mothers. This could be explained by different reasons, such as a long-distance seed dispersal event, previous conservation actions, their closed genetic distance and/or the noise effects of missing data during analysis.

From all the studied seedlings and saplings, most (103 of 109; 94.5%) were originated by self-fertilization, being the other six (11.2.P, 11.3.P, 11.7.P, 11.8.P, 18.2.P and 18.6.P) originated by xenogamy (Table 3). For six plants (16.2.P, 16.3.P, 18.10.P, 18.13.P, 20.1.P and 22.18.P), only one of the progenitors in the population was not inferred and consequently they are putative hybrids with other *Abies* species. To shed light on the possible hybrid origin of these individuals, we carried out a Principal Component Analysis (PCA) using 95 loci (including 18 out 20 selected for this issue) present in *A. nebrodensis*, *A. cephalonica* and *A. alba*. The PCA clearly separated the three species, being the positive values on the X-axis represented by *A. nebrodensis* plants (Figure 5A). Of the six potential hybrids, **two of them (18.10.P and 20.1.P) seem to be genetically close to *A. alba* and *A. cephalonica*, respectively, so they can be considered hybrids.** The remaining four plants were assigned as *A. nebrodensis*. In addition, according to the PCA, those plants with an uncertain origin (i.e. 21.3.P, 21.4.P and 21.5.P) were genetically similar to *A. nebrodensis*, despite their origin could not be associated to any adult tree currently occurring in the population.

Furthermore, a surprisingly result was obtained for the adult plants 31M and 21 M. The 31M individual was framed within the cloud of points of *A. cephalonica* and plant 21M seems to be genetically more similar to *A. alba* rather than *A. nebrodensis*. **That is, 31M and 21M**

seems to have a hybrid origin. In addition, three seedlings labeled as offspring of plant 21M were not correctly assigned either to their putatively mother plant nor any other adult tree of *A. nebrodensis*. This definitely increases our suspicious that **these three seedlings may be hybrids with other fir species**. Finally, a DAPC was performed to investigate the hybrid origin of seedlings.

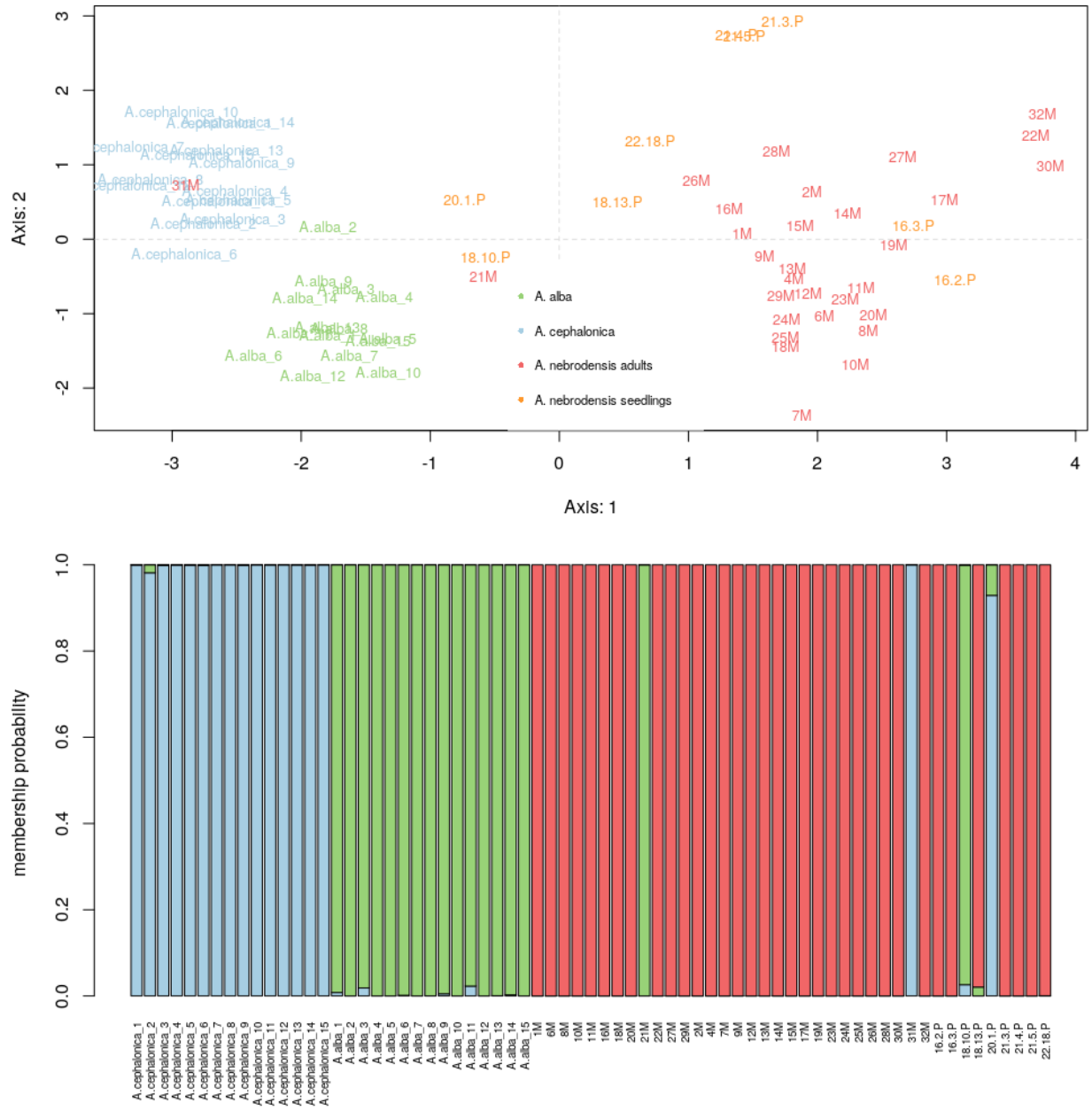

Figure 5. (A) Two-dimensional scatterplot extracted from the Principal Component Analysis (PCA) of *A. cephalonica* (blue), *A. alba* (green), adult trees of *A. nebrodensis* (red) and seedlings from natural regeneration with a putative hybrid origin (orange). The first two factors explained 22.4% and 6.3% of the total variation, respectively. (B) Barplot based on a discriminant analysis of principal components (DAPC) of the genetic information from 95 loci sites, in which the assignment probability to each of the three genetic clusters (red, green and blue) of individuals of *A. cephalonica*, *A. alba* and *A. nebrodensis* (adults and seedlings) is represented.

Again, we found the existence of three genetic clusters that agree with taxonomic classification (i.e. species), except for plants 21M and 31M and for seedlings 18.10.P and 20.1.P, which were appeared in the *A. alba* and *A. cephalonica* groups.

8. Conclusions

We found that most adult tree in the natural population are genetically close. Effective population size (N_e), a key parameter in population genetics to estimate the number of individuals that effectively contributes offspring to the next generation, was markedly low (only 6).

Most seedlings/saplings from natural regenerations are "pure *A. nebrodensis*" but mainly originated by self-fertilization. Genetic drift and inbreeding are expected to decrease the ability of seedlings to cope with a wide variety of environmental stressors, which probably would explain the low survival rate of seedlings reported in previous studies.

On the other hand, we can consider **very likely the hybrid origin of the 18.10.P and 20.1.P seedlings, whereas 21M and 31M adults, are also suspicious to be non-pure *A. nebrodensis*** or even alien fir species (especially in the case of 31M). Likewise, plants without a complete assignment to their parental origin (16.2.P, 16.3.P, 18.13.P, 21.3.P, 21.4.P, 21.5.P and 22.18.P), or without assignment (21.3.P, 21.4.P and 21.5.P), should be careful considered as potential hybrids, even though evidences from PCA suggest that they probably are *A. nebrodensis* individuals.

9. *Ex situ* conservation of DNA at the DNA bank of the University of Seville

Vegetal material (leaves) collected from adult tress of *A. nebrodensis* were deposited in the DNA bank of the University of Seville for the long-term conservation. In particular, the DNA bank will harbor material from 29 of 30-adult trees (all plants except for 20M; Table 4). The amount of vegetal material available may vary depending on the material used during the DNA extractions. Samples from each individual are given a unique accession number in the herbarium database. A voucher specimen of *A. nebrodensis* is available at the Servicio General de Investigacion de Herbario (SEV), Universidad (voucher: SEV256497).

Tables

Table 1. Genetic diversity estimations of adult trees. *PHt* (proportion of heterozygous loci in an individual), *Hs* (Standardized heterozygosity based on the mean expected heterozygosity), *IR* (internal relatedness), *HL* (homozygosity by locus) and *INBR* (inbreeding coefficient). Colors of coefficients range from lower (dark blue) to high values (yellow).

	PHt	Hs_exp	IR	HL	INBR
13M	0.49	1.13	-0.12	0.50	-0.20
22M	0.42	0.97	0.02	0.57	-0.02
29M	0.42	0.97	0.01	0.57	-0.01
12M	0.40	0.93	0.13	0.57	-0.01
14M	0.40	0.93	0.09	0.59	0.02
26M	0.40	0.93	0.08	0.58	0.04
10M	0.39	0.90	0.13	0.61	0.17
18M	0.39	0.90	0.11	0.61	0.12
11M	0.36	0.83	0.15	0.61	0.11
17M	0.36	0.83	0.17	0.63	0.10
20M	0.35	0.81	0.15	0.63	0.06
23M	0.34	0.79	0.16	0.63	0.07
8M	0.34	0.79	0.29	0.64	0.57
2M	0.33	0.76	0.23	0.65	0.15
9M	0.33	0.76	0.23	0.66	0.12
15M	0.32	0.74	0.22	0.67	0.08
24M	0.32	0.74	0.22	0.66	0.13
25M	0.32	0.74	0.23	0.66	0.18
4M	0.31	0.72	0.28	0.67	0.25
6M	0.30	0.69	0.27	0.68	0.20
7M	0.30	0.69	0.31	0.69	0.20
28M	0.28	0.65	0.34	0.71	0.17
1M	0.26	0.60	0.37	0.74	0.25
27M	0.26	0.60	0.39	0.73	0.31
21M	0.25	0.58	0.44	0.75	0.55
32M	0.25	0.58	0.45	0.74	0.36
30M	0.21	0.49	0.54	0.78	0.61
19M	0.18	0.42	0.63	0.83	0.79
16M	0.16	0.37	0.59	0.83	0.40
31M	0.14	0.32	0.68	0.86	0.65

Table 2. List of 30 recommended crosses between mature adult trees of *A. nebrodensis* ordered by more distant co-ancestry and, therefore, more convenient crosses to increase genetic diversity. Plants with a suspicious origin were highlighted with red font. Please note some of the recommended outcrossing may involve non-reproductive individuals.		
Cross 1	19M	26M
Cross 2	11M	19M
Cross 3	17M	31M
Cross 4	9M	30M
Cross 5	31M	32M
Cross 6	21M	30M
Cross 7	7M	32M
Cross 8	8M	9M
Cross 9	8M	19M
Cross 10	16M	30M
Cross 11	10M	27M
Cross 12	30M	31M
Cross 13	22M	7M
Cross 14	7M	30M
Cross 15	25M	28M
Cross 16	21M	22M
Cross 17	1M	22M
Cross 18	18M	9M
Cross 19	27M	25M
Cross 20	1M	29M
Cross 21	8M	12M
Cross 22	24M	28M
Cross 23	4M	25M
Cross 24	25M	26M
Cross 25	2M	14M
Cross 26	21M	32M
Cross 27	13M	15M
Cross 28	27M	24M
Cross 29	10M	30M
Cross 30	19M	28M

Table 3. List of the 118-juvenile individuals of *A. nebrodensis* originated from natural regeneration with their inferred progenitors as a result of paternity tests and the probability of correct assignment. Seedlings originated by xenogamy (column "Self-fertilization") were highlighted in bold. The probability of hybrid origin is also indicated.

Offspring ID	Inferred Dad	Inferred Mum	Probability (%)	Self-fertilization	Hybrid origin
1.1.P	1M	1M	100	Yes	-
1.2.P	1M	1M	100	Yes	-
1.3.P	1M	1M	100	Yes	-
1.4.P	1M	1M	100	Yes	-
1.5.P	1M	1M	100	Yes	-
1.6.P	1M	1M	100	Yes	-
1.7.P	1M	1M	100	Yes	-
1.8.P	1M	1M	83.48	Yes	-
1.9.P	1M	1M	100	Yes	-
1.10.P	1M	1M	100	Yes	-
1.11.P	1M	1M	100	Yes	-
6.1.P	6M	6M	100	Yes	-
6.2.P	6M	6M	100	Yes	-
8.1.P	8M	8M	100	Yes	-
8.2.P	8M	8M	100	Yes	-
10.1.P	11M	11M	100	Yes	-
10.2.P	10M	10M	100	Yes	-
10.3.P	10M	10M	100	Yes	-
11.1.P	10M	10M	100	Yes	-
11.2.P	10M	11M	100	**No**	-
11.3.P	10M	11M	100	**No**	-
11.4.P	10M	10M	100	Yes	-
11.5.P	10M	10M	100	Yes	-
11.6.P	10M	10M	100	Yes	-
11.7.P	10M	11M	40.44	**No**	-
11.8.P	10M	11M	42.66	**No**	-
11.9.P	10M	10M	100	Yes	-
11.10.P	17M	17M	100	Yes	-
11.11.P	2M	2M	100	Yes	-
11.12.P	1M	1M	100	Yes	-
11.13.P	8M	8M	100	Yes	-
11.14.P	10M	10M	100	Yes	-
11.15.P	10M	10M	100	Yes	-
11.16.P	10M	10M	100	Yes	-
11.17.P	10M	10M	100	Yes	-
11.18.P	17M	17M	100	Yes	-
11.19.P	23M	23M	100	Yes	-
16.1.P	17M	17M	100	Yes	-
16.2.P	Unknown	17M	74.58	Unknown	Probable
16.3.P	Unknown	17M	74.61	Unknown	Probable
18.1.P	18M	18M	100	Yes	-
18.2.P	22M	18M	100	**No**	-
18.3.P	18M	18M	100	Yes	-
18.4.P	18M	18M	100	Yes	-
18.5.P	18M	18M	100	Yes	-

18.6.P	17M	18M	100	**No**	-
18.7.P	18M	18M	100	Yes	-
18.8.P	18M	18M	100	Yes	-
18.9.P	18M	18M	100	Yes	-
18.10.P	Unknown	18M	100	Unknown	Very high
18.11.P	18M	18M	100	Yes	-
18.12.P	18M	18M	100	Yes	-
18.13.P	Unknown	18M	100	Unknown	Probable
18.14.P	18M	18M	100	Yes	-
18.15.P	18M	18M	100	Yes	-
18.16.P	18M	18M	100	Yes	-
18.17.P	18M	18M	100	Yes	-
20.1.P	Unknown	29M	62.01	Unknown	Very high
20.2.P	29M	29M	100	Yes	-
20.3.P	29M	29M	100	Yes	-
20.4.P	29M	29M	100	Yes	-
21.3.P	Unknown	Unknown	100	Unknown	Very high
21.4.P	Unknown	Unknown	100	Unknown	Very high
21.5.P	Unknown	Unknown	100	Unknown	Very high
22.1.P	22M	22M	100	Yes	-
22.2.P	22M	22M	100	Yes	-
22.3.P	22M	22M	100	Yes	-
22.4.P	22M	22M	100	Yes	-
22.5.P	22M	22M	100	Yes	-
22.6.P	22M	22M	100	Yes	-
22.7.P	22M	22M	100	Yes	-
22.8.P	22M	22M	96.28	Yes	-
22.9.P	22M	22M	100	Yes	-
22.10.P	22M	22M	100	Yes	-
22.11.P	22M	22M	100	Yes	-
22.12.P	22M	22M	100	Yes	-
22.13.P	22M	22M	100	Yes	-
22.14.P	22M	22M	100	Yes	-
22.15.P	22M	22M	100	Yes	-
22.16.P	22M	22M	100	Yes	-
22.17.P	22M	22M	100	Yes	-
22.18.P	Unknown	22M	100	Unknown	Probable
22.19.P	22M	22M	100	Yes	-
22.20.P	22M	22M	100	Yes	-
22.21.P	22M	22M	100	Yes	-
22.22.P	22M	22M	100	Yes	-
22.23.P	22M	22M	100	Yes	-
22.24.P	22M	22M	100	Yes	-
22.25.P	22M	22M	100	Yes	-
22.26.P	22M	22M	100	Yes	-
22.27.P	22M	22M	100	Yes	-
22.28.P	22M	22M	100	Yes	-
22.29.P	22M	22M	100	Yes	-
22.30.P	22M	22M	100	Yes	-
22.31.P	22M	22M	100	Yes	-

22.32.P	22M	22M	100	Yes	-
22.33.P	22M	22M	100	Yes	-
22.34.P	22M	22M	100	Yes	-
22.35.P	22M	22M	100	Yes	-
22.36.P	22M	22M	100	Yes	-
22.37.P	22M	22M	100	Yes	-
22.38.P	22M	22M	100	Yes	-
22.39.P	22M	22M	100	Yes	-
22.40.P	22M	22M	100	Yes	-
22.41.P	22M	22M	100	Yes	-
22.42.P	22M	22M	100	Yes	-
27.1.P	27M	27M	100	Yes	-
27.2.P	27M	27M	100	Yes	-
27.3.P	27M	27M	100	Yes	-
29.1.P	29M	29M	100	Yes	-
29.2.P	29M	29M	100	Yes	-
29.3.P	29M	29M	100	Yes	-
29.4.P	29M	29M	100	Yes	-
29.5.P	29M	29M	100	Yes	-
29.6.P	29M	29M	100	Yes	-
29.7.P	29M	29M	100	Yes	-
29.8.P	29M	29M	100	Yes	-
23.1.P	23M	23M	100	Yes	-

Table 4. List of accession numbers of the vegetal material from 29 of the 30-adult trees of *A. nebrodensis* deposited in the DNA bank of the University of Seville. Identification of adult trees correspond to original nomenclature.

ID number of adult trees according to Virgilio et al. 2000 and posterior studies	**Accession number assigned in the DNA Bank of the University of Seville**
1M	599
2M	600
4M	601
6M	602
7M	603
8M	604
9M	605
10M	606
11M	607
12M	608
13M	609
14M	610
15M	611
16M	612
17M	613
18M	614
19M	615
21M	616
22M	617
23M	618
24M	619
25M	620
26M	621
27M	622
28M	623
29M	624
30M	625
31M	626
32M	627

Supplementary information

Table S1. Ritland estimator (*RIT*) of pairwise co-ancestry between adult trees of *A. nebrodensis* ordered by negative values.		
19M	26M	-0.3837
11M	19M	-0.3818
17M	31M	-0.3807
9M	30M	-0.3757
31M	32M	-0.3735
21M	30M	-0.3675
7M	32M	-0.3645
8M	9M	-0.3582
8M	19M	-0.3569
16M	30M	-0.3543
10M	27M	-0.3454
30M	31M	-0.3307
22M	7M	-0.3251
7M	30M	-0.3095
25M	28M	-0.3090
21M	22M	-0.3078
1M	22M	-0.3067
18M	9M	-0.2982
27M	25M	-0.2889
1M	29M	-0.2882
8M	12M	-0.2843
24M	28M	-0.2836
4M	25M	-0.2822
25M	26M	-0.2809
2M	14M	-0.2739
21M	32M	-0.2668
13M	15M	-0.2667
27M	24M	-0.2639
10M	30M	-0.2605
19M	28M	-0.2603
18M	32M	-0.2585
8M	27M	-0.2580
4M	24M	-0.2574
24M	26M	-0.2560
1M	18M	-0.2539
16M	29M	-0.2519
16M	32M	-0.2515
19M	25M	-0.2507
22M	31M	-0.2500

29M	4M	-0.2481
10M	28M	-0.2458
27M	12M	-0.2441
6M	28M	-0.2433
14M	31M	-0.2432
6M	18M	-0.2381
11M	27M	-0.2359
1M	32M	-0.2348
26M	32M	-0.2334
10M	31M	-0.2320
10M	21M	-0.2313
10M	15M	-0.2311
27M	9M	-0.2295
12M	17M	-0.2274
16M	22M	-0.2270
19M	24M	-0.2256
29M	26M	-0.2247
2M	7M	-0.2221
20M	14M	-0.2213
17M	19M	-0.2195
15M	30M	-0.2179
21M	23M	-0.2150
6M	14M	-0.2130
8M	28M	-0.2123
1M	19M	-0.2112
12M	28M	-0.2104
26M	30M	-0.2097
8M	31M	-0.2094
19M	23M	-0.2072
22M	9M	-0.2064
8M	16M	-0.2059
10M	17M	-0.2051
27M	29M	-0.2036
2M	4M	-0.1982
9M	23M	-0.1940
18M	2M	-0.1929
17M	23M	-0.1922
1M	20M	-0.1914
19M	30M	-0.1906
15M	25M	-0.1903
8M	22M	-0.1885
6M	8M	-0.1873

23M	28M	-0.1856
24M	32M	-0.1854
25M	32M	-0.1854
21M	27M	-0.1828
8M	15M	-0.1820
1M	10M	-0.1798
20M	30M	-0.1792
21M	25M	-0.1763
4M	7M	-0.1759
29M	30M	-0.1756
2M	15M	-0.1750
19M	31M	-0.1708
6M	27M	-0.1689
15M	19M	-0.1686
7M	31M	-0.1666
15M	24M	-0.1657
16M	18M	-0.1643
12M	32M	-0.1643
11M	32M	-0.1640
7M	26M	-0.1640
20M	32M	-0.1628
27M	23M	-0.1606
18M	21M	-0.1605
9M	32M	-0.1602
16M	20M	-0.1590
11M	4M	-0.1568
1M	2M	-0.1535
21M	24M	-0.1520
27M	31M	-0.1506
29M	17M	-0.1467
6M	17M	-0.1459
21M	12M	-0.1459
18M	31M	-0.1458
20M	31M	-0.1445
6M	13M	-0.1443
4M	26M	-0.1442
13M	30M	-0.1437
21M	14M	-0.1429
9M	14M	-0.1390
22M	25M	-0.1387
27M	2M	-0.1373
29M	9M	-0.1367

1M	21M	-0.1340
11M	21M	-0.1337
6M	32M	-0.1310
11M	14M	-0.1301
16M	19M	-0.1296
7M	17M	-0.1287
24M	30M	-0.1278
8M	2M	-0.1276
29M	28M	-0.1241
10M	22M	-0.1225
16M	17M	-0.1189
22M	26M	-0.1177
22M	19M	-0.1157
22M	24M	-0.1144
14M	25M	-0.1144
16M	21M	-0.1127
23M	26M	-0.1103
1M	30M	-0.1102
6M	20M	-0.1102
11M	28M	-0.1101
20M	9M	-0.1096
23M	30M	-0.1088
16M	12M	-0.1080
1M	17M	-0.1070
6M	29M	-0.1061
13M	25M	-0.1049
2M	28M	-0.1047
20M	23M	-0.1045
2M	23M	-0.1045
8M	4M	-0.1037
21M	4M	-0.1023
12M	13M	-0.1022
11M	16M	-0.1021
10M	26M	-0.1020
6M	23M	-0.0998
2M	26M	-0.0992
20M	22M	-0.0991
19M	32M	-0.0985
21M	19M	-0.0984
1M	4M	-0.0980
9M	17M	-0.0977
18M	27M	-0.0974

23M	32M	-0.0970
6M	22M	-0.0964
2M	30M	-0.0955
10M	25M	-0.0933
23M	25M	-0.0927
27M	14M	-0.0904
14M	24M	-0.0890
6M	30M	-0.0883
9M	13M	-0.0848
22M	15M	-0.0837
16M	4M	-0.0804
13M	24M	-0.0804
14M	15M	-0.0776
17M	25M	-0.0775
18M	28M	-0.0773
18M	30M	-0.0773
2M	17M	-0.0769
22M	23M	-0.0762
12M	15M	-0.0759
7M	25M	-0.0754
23M	31M	-0.0748
16M	15M	-0.0738
16M	28M	-0.0728
18M	17M	-0.0727
11M	20M	-0.0711
13M	19M	-0.0710
18M	19M	-0.0701
4M	12M	-0.0700
10M	4M	-0.0692
6M	26M	-0.0691
10M	24M	-0.0690
10M	32M	-0.0688
9M	28M	-0.0688
13M	17M	-0.0688
23M	24M	-0.0683
22M	13M	-0.0682
4M	13M	-0.0682
11M	18M	-0.0661
11M	25M	-0.0659
7M	28M	-0.0656
1M	28M	-0.0620
29M	2M	-0.0611

14M	17M	-0.0604
8M	25M	-0.0603
12M	24M	-0.0601
6M	19M	-0.0600
20M	21M	-0.0594
20M	25M	-0.0587
6M	15M	-0.0584
11M	22M	-0.0565
4M	32M	-0.0565
7M	14M	-0.0565
13M	26M	-0.0541
1M	13M	-0.0539
17M	24M	-0.0529
4M	9M	-0.0517
1M	8M	-0.0515
7M	24M	-0.0501
22M	12M	-0.0500
1M	15M	-0.0496
7M	19M	-0.0489
6M	10M	-0.0487
18M	22M	-0.0474
20M	4M	-0.0470
10M	16M	-0.0452
21M	13M	-0.0441
9M	15M	-0.0437
11M	17M	-0.0435
13M	31M	-0.0431
11M	24M	-0.0416
14M	26M	-0.0416
8M	18M	-0.0406
29M	31M	-0.0399
15M	31M	-0.0369
8M	24M	-0.0358
29M	13M	-0.0358
13M	32M	-0.0354
20M	24M	-0.0344
1M	14M	-0.0337
10M	12M	-0.0315
6M	7M	-0.0311
28M	31M	-0.0300
13M	23M	-0.0294
8M	14M	-0.0289

4M	17M	-0.0285
16M	25M	-0.0260
11M	15M	-0.0259
4M	14M	-0.0255
16M	26M	-0.0253
10M	7M	-0.0236
10M	14M	-0.0233
28M	30M	-0.0230
1M	26M	-0.0225
25M	31M	-0.0212
22M	29M	-0.0192
11M	29M	-0.0182
11M	7M	-0.0175
11M	30M	-0.0164
10M	2M	-0.0160
20M	15M	-0.0160
16M	27M	-0.0146
10M	13M	-0.0142
8M	32M	-0.0140
22M	4M	-0.0135
16M	2M	-0.0131
18M	25M	-0.0126
15M	32M	-0.0125
17M	30M	-0.0113
21M	28M	-0.0109
12M	26M	-0.0090
18M	26M	-0.0077
4M	30M	-0.0069
6M	31M	-0.0067
10M	20M	-0.0065
27M	13M	-0.0053
1M	12M	-0.0052
22M	28M	-0.0031
27M	7M	-0.0023
8M	7M	-0.0020
16M	24M	-0.0017
29M	23M	-0.0015
13M	28M	-0.0010
2M	12M	-5.00E-04
1M	11M	6.00E-04
16M	13M	0.0018
25M	30M	0.0018

11M	2M	0.0029
21M	9M	0.0032
24M	31M	0.0034
11M	13M	0.0046
11M	31M	0.0057
1M	7M	0.0076
20M	2M	0.0091
6M	4M	0.0099
14M	32M	0.0102
8M	17M	0.0110
8M	30M	0.0112
8M	29M	0.0113
10M	18M	0.0113
18M	24M	0.0117
10M	11M	0.0122
16M	14M	0.0128
20M	28M	0.0137
9M	12M	0.0150
18M	15M	0.0162
20M	27M	0.0168
21M	2M	0.0170
20M	19M	0.0175
29M	32M	0.0227
15M	23M	0.0228
8M	11M	0.0242
6M	16M	0.0271
22M	14M	0.0288
8M	20M	0.0290
29M	25M	0.0297
1M	9M	0.0306
7M	12M	0.0319
7M	9M	0.0334
21M	17M	0.0352
2M	19M	0.0364
1M	23M	0.0377
20M	29M	0.0377
12M	14M	0.0387
12M	19M	0.0388
20M	13M	0.0394
1M	27M	0.0396
17M	26M	0.0398
29M	15M	0.0406

27M	19M	0.0440
9M	26M	0.0468
12M	31M	0.0500
11M	9M	0.0516
29M	12M	0.0517
1M	6M	0.0533
29M	24M	0.0540
16M	9M	0.0549
6M	2M	0.0551
18M	12M	0.0555
6M	12M	0.0556
18M	20M	0.0593
7M	13M	0.0594
1M	24M	0.0607
4M	31M	0.0611
12M	30M	0.0617
16M	7M	0.0642
6M	21M	0.0647
2M	13M	0.0667
4M	28M	0.0670
14M	23M	0.0682
15M	26M	0.0697
4M	15M	0.0709
2M	25M	0.0716
18M	29M	0.0721
10M	9M	0.0746
12M	25M	0.0766
4M	23M	0.0778
11M	23M	0.0795
12M	23M	0.0827
20M	26M	0.0830
9M	24M	0.0856
9M	25M	0.0856
14M	28M	0.0860
28M	32M	0.0862
29M	14M	0.0882
27M	26M	0.0895
16M	31M	0.0901
21M	7M	0.0916
21M	29M	0.0917
20M	12M	0.0919
22M	2M	0.0952

18M	14M	0.0959
2M	24M	0.0967
1M	31M	0.0968
6M	24M	0.1013
21M	15M	0.1035
22M	17M	0.1038
7M	23M	0.1080
29M	7M	0.1096
7M	15M	0.1116
29M	19M	0.1119
8M	26M	0.1162
18M	4M	0.1168
2M	32M	0.1184
27M	4M	0.1303
9M	31M	0.1345
10M	23M	0.1386
20M	7M	0.1397
13M	14M	0.1405
9M	19M	0.1455
11M	12M	0.1466
27M	32M	0.1499
8M	10M	0.1519
6M	25M	0.1526
18M	23M	0.1545
8M	21M	0.1553
27M	30M	0.1561
2M	31M	0.1570
21M	26M	0.1614
16M	23M	0.1698
22M	27M	0.1716
8M	13M	0.1752
18M	13M	0.1782
17M	28M	0.1873
1M	25M	0.1890
14M	19M	0.1969
27M	17M	0.2032
6M	11M	0.2059
14M	30M	0.2115
8M	23M	0.2139
26M	31M	0.2148
15M	17M	0.2196
11M	26M	0.2297

17M	32M	0.2406
15M	28M	0.2480
2M	9M	0.2494
20M	17M	0.2766
27M	15M	0.2829
18M	7M	0.2838
10M	29M	0.3243
6M	9M	0.4061
26M	28M	0.4244
10M	19M	0.4541
4M	19M	0.5319
21M	31M	0.5353
27M	28M	0.5591
30M	32M	0.7385
22M	32M	0.8053
1M	16M	0.8810
22M	30M	0.8894
24M	25M	1.0587