Article

Genetic Variability of *Alnus cordata* (Loisel.) Duby Populations and Introgressive Hybridization with *A. glutinosa* (L.) Gaertn. in Southern Italy: Implication for Conservation and Management of Genetic Resources

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Abstract: *Alnus cordata* (Loisel.) Duby (Neapolitan alder) is an endemic tree species with a restricted distribution range, limited to Corsica and southern Italy. The economic value of its wood, its rapid growth, the tolerance to drought stress and the nitrogen fixation capacity make *A. cordata* an excellent candidate for breeding, as well as for conservation and management of genetic resources. In this context, we evaluated the genetic variability of southern Italy populations and verified the hybridization capacity with the simpatric species *A. glutinosa* (L.) Gaertn. Eight pure *A. cordata* populations, two pure *A. glutinosa* populations and six mixed *A. cordata*/*A. glutinosa* populations located in southern Italy were analyzed using seven microsatellite markers. A low genetic diversity within and among populations was observed, but no inbreeding effects were evident. A variable frequency of F_2 interspecific hybrids was observed in most of the mixed populations and few backcross individuals were scored. These results suggest a limited capacity of hybrid individuals to cross back with the parent species, reducing the risk of genetic pollution of *A. cordata*. This work provides meaningful knowledge for the conservation and management of the endemic species *A. cordata,* which represents a valuable source of biodiversity to be conserved.

Keywords: alder; *Alnus* spp.; backcross; genetic diversity; hybridization; genetic conservation; introgression; speciation genes

1. Introduction

Hybridization plays an important role in evolution, speciation and even species extinction [1]. An increased rate of hybridization with related and more abundant species could represent a threat to the survival of rare and endemic species. Moreover, extensive hybridization of a widely-distributed species with endemic ones may result in "genetic swamping" or "demographic swamping" and can wipe out the endemic species [2–4]. Several studies have documented the decline of rare plant species due to hybridization phenomena [5–9].

The genus *Alnus* (Mill.) (Betulaceae), commonly referred as alder, includes monoecious trees and shrubs widely-distributed throughout the temperate zone of the northern Hemisphere, with a few species extending in central America as well as in the northern and southern Andes. Studies on population genetics and phylogeography have been carried out on different *Alnus* species, i.e., *A. rubra* Bong [10,11], *A. maritima* (Marshall) Muhl. ex Nutt [12,13], *A. serrulata* Willd. [13], *A. glutinosa* (L.) Gaertn. [14–17], *A. rugosa* Spreng. [18], *Alnus alnobetula* subsp. *crispa* (Aiton) Raus [19] and *A. incana* (L.) Moench [20]; moreover,

natural hybridization and introgression between *Alnus* species have been recorded and well documented, including hybrids of *A. glutinosa* × *incana* [21–24], *A. serrulata* (Aiton) Willd. × *A. incana* subsp. rugosa (Du Roi) [25], and *A. glutinosa* × *A. rubra* Bong. [26], *A. glutinosa* × *A. rohlenae* Vít. [27].

Four species of *Alnus* are present in Italy: *A. incana* (L.), *A. glutinosa*, *A. alnobetula* (Ehrh.) K.Koch subsp. *alnobetula* (L.) and *A. cordata* (Loisel.) Duby. *A. cordata*, known as "Neapolitan alder", is an endemic species that grows in small areas of the southern Apennines, and mountains of north-eastern Corsica [28]. In southern Italy and in other areas of sympatry, *A. cordata* shares its natural distribution range with *A. glutinosa*, species widespread throughout Europe. Despite its limited natural range, *A. cordata* is not considered an endangered species. This species has experienced little human intervention; its most important threats are the competition with other species, the isolation due to reduction and/or absence of gene flow between populations and the isotherm shift in the Mediterranean region due to climate change [28]. Literature on *A. cordata* is rather scarce, but previous studies highlighted its excellent tolerance to drought stress, a relevant feature to face climatic changes [29,30]. Previous research [31] on *A. cordata* and *A. glutinosa* populations of southern Italy and Corsica highlighted different gene pools and hypothesized paleo-introgression events between the two species. *A.cordata* and *A. glutinosa* show strong phenotypic similarities [31], but little information is available on hybrids. Their interspecific hybrid, named *A.* × *elliptica* Req., was observed in Corsica, with rare individuals identified by leaf morphology [15], but no reports are available for sympatric areas of southern Italy.

Starting from the 1980s, considerable attention has been paid to the management, and genetic improvement of the genus *Alnus* [10,32] due to the economic value of its wood [33,34], its rapid growth and its nitrogen fixation capacity conferred by the symbiotic association with the soil actinomycete *Frankia* (Actinomycetales). All these features also made alder an interesting option for bioremediation, "energy plantation systems" and for promoting the growth of other species and the ecosystem development [14,26,35,36]. Moreover, *A. cordata* ecosystems showed N-rich litter, large organic C and total N stock indicating this species as ideal for afforestation and reforestation [37]. Given its particular traits, *A. cordata* has gained interest as genetic resources in breeding programs, with the aim of transferring its favorable characteristics to other *Alnus* species and obtain superior interspecific hybrids [26]. In this context, the management and conservation of endemic genetic resources as *A. cordata* require the evaluation of genetic variability of the species, the evaluation of the presence of hybrids and the understanding of the factors that contribute to the destructive outcomes versus the constructive ones of hybridization.

The main objectives of this study were (1) to evaluate the genetic variability of natural populations of *A. cordata* in its major natural distribution range, (2) to identify hybridization phenomena with *A. glutinosa* in areas of sympatry and (3) to evaluate whether hybridization and introgression processes may threat the genetic integrity of *A. cordata*.

2. Materials and Methods

A total of 319 individuals were collected from sampling sites in southern Italy, in the regions of Campania, Basilicata and Calabria (Figure 1), according to the distribution range of *A. cordata* and *A. glutinosa* [28] and the census made by Regional Forestry Agency. We analyzed 16 natural populations, consisting of eight pure *A. cordata*, six mixed *A. cordata/A. glutinosa* and two pure *A. glutinosa* found in the area where the presence of *A. cordata* is fragmented and restricted to few sites. Based on the population size, 16 to 26 trees per site were sampled and georeferenced (Table 1, Figure 1).
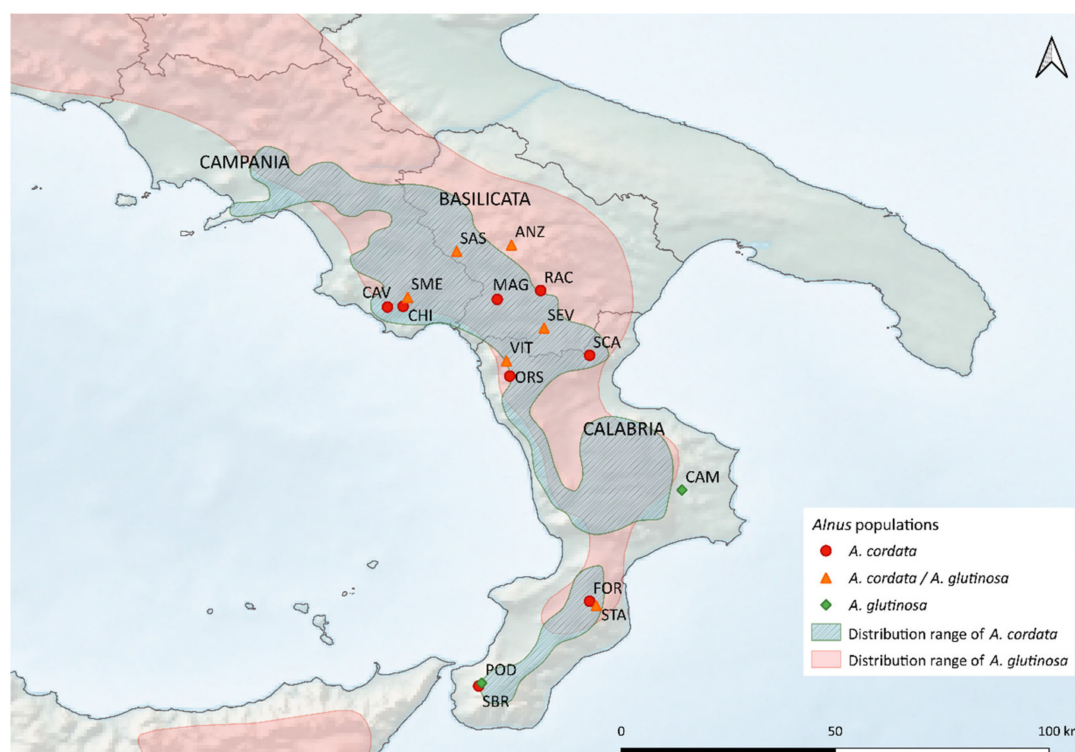
Figure 1. Distribution range (Euforgen. Available online http://www.euforgen.org/species/, accessed on 20 May 2021) of *A. cordata* and *A. glutinosa* and sampling sites of the 16 populations analyzed in this study.

Table 1. Geographic and sampling information of 16 *A. cordata* and *A. glutinosa* populations: population code (Pop ID), sampling site (Municipality), province (Pr), geographic coordinates in decimal degrees, latitude (Lat.) and longitude (Long.), elevation above sea level (Elev.), number of individuals sampled (Ni). The last three columns present the number of individuals with morphological assignment to each species or to uncertain classification.

Pop ID	Municipality	Pr	Lat.	Long.	Elev. (m)	Ni	*A. cordata*	*A. glutinosa*	*Uncert.*
				Campania Region					
CAV	Cuccaro Vetere	SA	40.1613	15.2946	680	19	19	0	0
CHI	Montano Antilia	SA	40.1645	15.3779	700	20	19	0	1
SME	Rofrano	SA	40.2130	15.4018	521	25	15	5	5
				Basilicata Region					
RAC	Gallicchio	PZ	40.2488	16.1076	370	18	18	0	0
SEV	Chiaromonte	PZ	40.0491	16.1255	630	20	13	7	0
MAG	Moliterno	PZ	40.2017	15.8772	740	20	20	0	0
ANZ	Anzi	PZ	40.4913	15.9526	585	16	13	3	0
SAS	Sasso di Castalda	PZ	40.4570	15.6617	740	24	15	6	3
				Calabria Region					
CAM	Santa Severina	KR	39.1889	16.8576	100	17	0	17	0
SCA	Plataci	CS	39.9044	16.3673	1156	18	18	0	0
VIT	Mormanno	CS	39.8745	15.9254	180	26	17	4	5
ORS	Papasidero	CS	39.7942	15.9435	220	19	19	0	0
FOR	Brognaturo	VV	38.5984	16.3674	950	19	19	0	0
STA	Brognaturo	VV	38.5751	16.4023	1018	22	6	2	14
SBR	S. Stefano in Aspromonte	RC	38.1487	15.7789	1000	20	20	0	0
POD	S. Stefano in Aspromonte	RC	38.1646	15.7941	616	16	0	16	0

The preliminary taxonomic classification of each sampled tree as *A. glutinosa* or *A. cordata* was performed in the field according to morphology of leaves and bark (Acta Plantarum. Available online http://www.actaplantarum.org, accessed on 20 May 2021). In *A. cordata*, leaves are ovate or circular-ovate, cordate at base and bark is smooth and greyish brown; in *A. glutinosa*, leaves are obovate to circular, wedge-shaped at base and bark is fissured and dark brown [31]. In sympatric areas, where *A. cordata* and *A. glutinosa* coexisted,

several individuals were classified as "uncertain", based on the difficult interpretation of leaf morphology (Table 1, Figure 2). Fresh leaves were collected from individual trees and subsequently stored at −20 °C for subsequent DNA extraction and analyses.
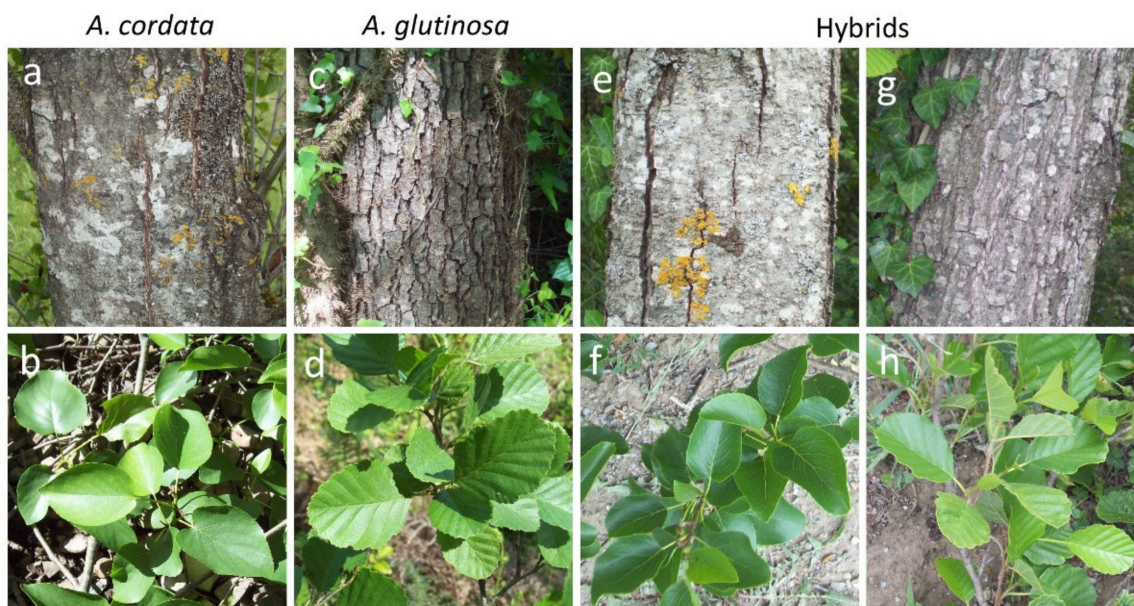

Figure 2. Examples of morphology of bark and leaves of *Alnus* trees sampled in this study. Taxonomic classification after NEWHYBRID genetic analysis: (**a**,**b**)*A. cordata*; (**c**,**d**) *A. glutinosa*; (**e**–**h**) interspecific hybrids.

2.1. DNA Isolation, SSR Amplification and Genotyping

Frozen leaves were ground to fine powder using liquid nitrogen. Up to 50 mg of ground tissue were used for DNA extraction with the DNeasy 96 Plant Kit (Qiagen, Valencia, CA, USA), according to the manufacturer's protocol. Twenty-one SSR markers, previously developed in *A. glutinosa* and *A. maritima* [38,39], were tested and seven of them, which gave polymorphic amplification products, were used for the analysis (Table S2). Two different PCR multiplex reactions were set up based on the amplicon size, using fluorescent dye-labelled primers (6-FAM, VIC, NED, PET; Applied Biosystems, Foster City, CA, USA). Amplifications were performed with the Type-It Microsatellite PCR Kit (Qiagen, Valencia, CA, USA). PCR mix consisted of 4 ng of genomic DNA, 1× Type-it Multiplex PCR Master Mix, 2 μM of each primer and RNase-free water for a total volume of 12.5 L. Amplification conditions were as follows: initial heat activation step at 95 °C for 5 min, followed by 27 cycles of denaturation step at 95 °C for 30 s, annealing step at 57 °C for 1.5 min, and extension step at 72 °C for 30 s. A final extension step at 60 °C for 30 min was executed. PCR fragments were run on an ABI PRIMS®3130 XL Genetic Analyzer for separation and sizing. GeneScan 250 LIZ was used as an internal size standard. Genotyping was performed using GeneMapper v4.0 software (Applied Biosystems, Foster City, CA, USA). The alleles were determined by automated binning and checked by visual inspection. The unbiased probability of identity (PIunb) [40] computed for the combination of the seven markers was PI = 0.00. This value indicates the probability that two unrelated trees selected at random from a population would have identical genotypes at multiple loci: the lower is this value, the higher is the capacity of the markers used to capture the variability present in the data set. The absence of null alleles was verified using FREENA software for each locus chosen for the analysis [41].

2.2. Genetic Validation and Assignment of Species and Hybrids

NEWHYBRID 1.1 software [42] was used to estimate the posterior probability that genetically sampled individuals fall into each of a set of user-defined hybrid categories.

NEWHYBRID assigned each individual to a different hybrid category, expressed in term of percentage of membership of each individual to a specific group. The categories in which individuals could fall are: parental (P0 = *A. cordata*; P1 = *A. glutinosa*); F1 and F2 hybrids, and two backcrosses (0Bx = backcross with *A. cordata* parental group; 1Bx = backcross with *A. glutinosa* parental group). Principal Coordinate Analysis (PCoA) for pure *A. cordata* and *A. glutinosa* populations and for hybrid mixed populations, based on genetic distance (GD), was performed using GeneAlEx 6.503 software [43]. Spatial analysis of NEWHYBRID results was performed using QGIS software [44], basing on a pie chart classification method.

2.3. Genetic Diversity and Population Structure

For each population, the number of alleles (N*a*), observed and expected heterozygosity (H*o*, H*e*), fixation index (Fis) and pairwise Fst were calculated using GeneAlex 6.503 software [43]. Allelic richness (Ar) was evaluated with HP-Rare 1.0 software [45]. The analysis of molecular variance (AMOVA) of the pure populations of *A. cordata* and *A. glutinosa* was calculated with Arlequin 3.1.1 software [46].

Population structure was inferred for all of the sampled populations using a Bayesian approach as implemented in STRUCTURE 2.3.4 software [47,48]. Analyses of population structure used admixture model with correlated allele frequencies. Parameters were set for a burn-in period of 100,000 and a MCMC (Markov chain Monte Carlo) with 200,000 iterations. Potential clusters (K) were tested using 20 iterations. To determine the most likely number of K, the ΔK method by Evanno et al. [49] was applied using STRUCTURE HARVESTER software [50]. A graphical representation of the STRUCTURE results was performed using CLUMPAK software [51].

3. Results

3.1. Genetic Validation and Assignment of Species and Hybrids

The analysis by NEWHYBRID allowed us to ascertain the species and hybrids assignment. Figure 3 presents the NEWHYBRID results as a map of the partitioning of individuals in 216 *A. cordata*, 71 *A. glutinosa* and 32 hybrids. The plants sampled in the eight populations of pure *A. cordata* showed an average degree of assignment of 98.6% to the P0 category (*A. cordata*) and genetically confirmed that all the individuals belonged to expected pure species. Similarly, the individuals sampled in the two pure *A. glutinosa* populations showed an average assignment of 98.9% to the category P1 (*A. glutinosa*) confirming, again, the expected botanic classification. The results of the mixed populations with sympatric species highlighted the presence of hybrids in all the *A. cordata*/*A. glutinosa* populations, except for the population "VIT". All the identified hybrids showed a high percentage of membership (mean of 79.5%) to the F_2 hybrid category, with no individuals assigned to F_1. Moreover, a low percentage of Backcross toward both parental species was found in all the mixed populations and never exceeded a percentage of assignment greater than 3% (Figure 3). The highest number of F_2 hybrids was identified in the population "SAS" (50%), while the population with the lowest value was "STA" (9.1%). An interesting exception was the mixed population "VIT", where, despite the co-presence of both species, there were no hybrids and all individuals belonged either to the species *A. cordata* (16 individuals) or *A. glutinosa* (10 individuals). Among the 28 individuals morphologically categorized as "Uncertain", because of the difficult interpretation of leaf morphology (Table 1), three were classified as *A. cordata*, 19 as *A. glutinosa* and six as hybrids.
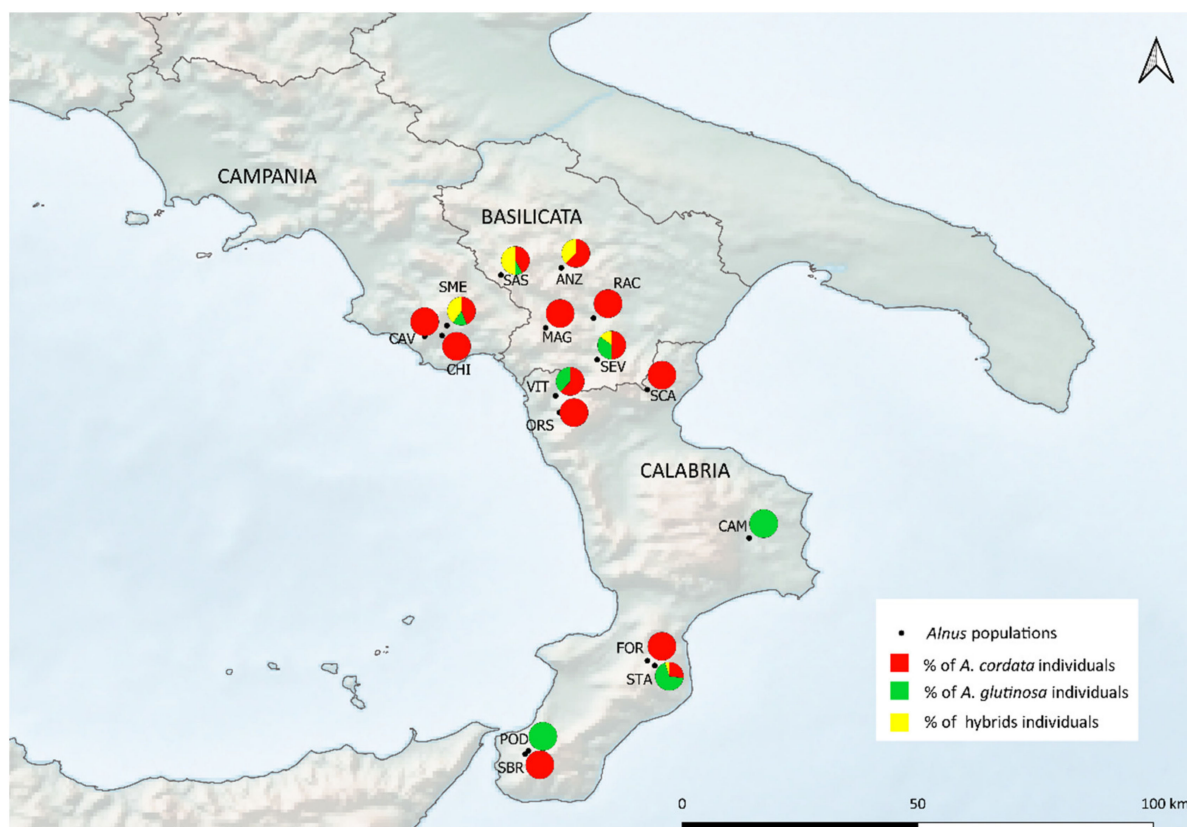
Figure 3. Partitioning of individuals in pure species *A. cordata* and *A. glutinosa*, hybrids and backcrosses, after NEWHYBRID genetic analysis of 16 *A. cordata* and *A. glutinosa* populations analyzed in this study. Pie diagrams show the percentage of individuals assigned to the different taxonomic categories in each population. Map created using QGIS 3.12 software.

3.2. Genetic Diversity and Population Structure

Genetic diversity indices were calculated for the three taxonomic groups obtained after NEWHYBRID assignment, i.e. *A. cordata*, *A. glutinosa* and hybrids (Table 2). The genetic diversity analysis of the 216 *A. cordata* individuals showed a mean value of Na = 5.571, observed heterozygosity (Ho) = 0.391 and a Fis = 0.190. The 71 individuals of *A. glutinosa* showed values of Na = 9.429, Ho = 0.533 and Fis = 0.203. Lastly, the 32 hybrid individuals showed values of Na = 8.429, Ho = 0.616 and Fis = 0.224. When comparing the two pure species, for all indices of genetic diversity, lower mean values were found in *A. cordata* compared to the *A. glutinosa*. However, the fixation index indicated a larger deficiency of heterozygosity in *A. glutinosa* in respect to *A. cordata*. When compared to the parent species, the hybrid individuals showed an intermediate number of alleles, but a higher value of heterozygosity and fixation index.

Table 2. Genetic diversity indices for the 319 individuals of *A. cordata*, *A. glutinosa* and hybrids.

Pop	N	Na	Ne	I	Ho	He	uHe	Fis
Alnus cordata (Loisel.) Duby	216	5.571	2.177	0.853	0.391	0.437	0.438	0.190
Alnus glutinosa (L.) Gaertn.	71	9.429	4.237	1.528	0.533	0.664	0.669	0.203
Hybrids	32	8.429	5.060	1.722	0.616	0.770	0.782	0.224

N = n° ofindividuals; Na = n° of alleles; Ne = expected n° of alleles; I = Shannon's index; Ho = observed heterozygosity; He = expected heterozygosity; uHe = unbiased expected heterozygosity; Fis = fixation index.

The genetic diversity indices were also calculated by single population (Table 3). Overall, the mixed populations of *A. cordata*/*A. glutinosa* showed higher values for the number of alleles (mean Na = 6.904) compared to pure populations of *A. cordata* (mean

Na = 3.107) and *A. glutinosa* (mean Na = 5.286). Higher values were also observed for heterozygosity in mixed populations compared to pure ones (mean Ho of 0.489, 0.396, 0.468 for mixed, *A. cordata* and *A. glutinosa*, respectively). All mixed populations showed significant positive Fis values, ranging from 0.237 to 0.461.

Table 3. Genetic diversity indices of 16 Alnus populations analyzed in this study.

Pop	Species	N	Na	Ne	Ho	He	uHe	Fis
CAV		19	3.143	2.074	0.436	0.452	0.465	0.098
CHI		20	3.286	2.001	0.436	0.439	0.450	0.035
RAC		18	3.143	2.060	0.436	0.421	0.434	−0.034
MAG		20	3.000	2.050	0.430	0.418	0.429	0.006
SCA	*Alnus cordata*	18	3.429	1.997	0.395	0.368	0.379	−0.088
ORS		19	3.000	2.014	0.308	0.379	0.389	0.243
FOR		19	2.857	2.064	0.391	0.378	0.389	−0.061
SBRU		20	3.000	1.930	0.343	0.358	0.368	0.188
SME		25	7.571	4.199	0.576	0.724	0.739	0.237 **
SEV		20	6.857	3.811	0.479	0.729	0.748	0.359 **
ANZ	Mixed *A. cordata*/*A. glutinosa*	16	6.143	3.365	0.509	0.653	0.674	0.263 **
SAS		24	7.286	3.949	0.523	0.699	0.714	0.290 **
VIT		25	6.714	3.587	0.391	0.688	0.702	0.461 **
STA		21	6.857	4.141	0.459	0.709	0.726	0.400 **
CAM	*Alnus glutinosa*	17	6.286	3.281	0.483	0.593	0.612	0.187 **
POD		16	4.286	2.793	0.514	0.546	0.565	0.039

N = *n°* of individuals; Na = *n°* of alleles; Ne = expected *n°* of alleles; *I* = Shannon's index; Ho = observed heterozygosity; He = expected heterozygosity; uHe = unbiased expected heterozygosity; Fis = fixation index. ** *p*<0.001.

Genetic diversity indices calculated for the single loci are reported in Table 4. F*is*, F*it* and F*st* indices were markedly variable and showed high values for the loci "alma1", "alng4" and "AG20" and low values for the loci "alma7" and "AG13". The index *Nm*, indirect estimator of gene flow, showed an inverse pattern of variation and ranged from 0.236 of locus "AG20" to 3.789 of locus "alma7". Additional data of differentiation between populations and species are provided in the supplementary Table S1, which presents the allelic frequency of single loci calculated per population and per taxonomic group.

Table 4. Genetic diversity indices for the seven SSR markers used in this study.

Locus	Fis	Fit	Fst	Nm
alma1	0.470	0.639	0.320	0.532
alma7	0.113	0.168	0.062	3.789
alma11	0.073	0.181	0.116	1.903
alng4	0.283	0.434	0.211	0.937
AG10	0.023	0.165	0.146	1.466
AG20	0.508	0.761	0.515	0.236
AG13	0.098	0.177	0.088	2.600
Mean	0.224	0.361	0.208	1.638

Ht = total expected heterozygosity; Ho = observed heterozygosity; He = expected heterozygosity; Fis = (Mean He−Mean Ho)/Mean He; Fit = (Ht−Mean Ho)/Ht; Fst = (Ht−Mean He)/Ht; Nm = [(1/Fst)−1]/4.

STRUCTURE analysis identified two core genetic groups (most likely number K = 2). The plot of STRUCTURE's results (Figure 4) highlighted a clear genetic distinction between pure *A. cordata* populations (core 'Group I'), and pure *A. glutinosa* populations (core "Group II"). The individuals of mixed populations of *A. cordata*/*A. glutinosa* showed a high level of admixture between the two core genetic groups, with varying level of kinship to the gene pools of the pure species.
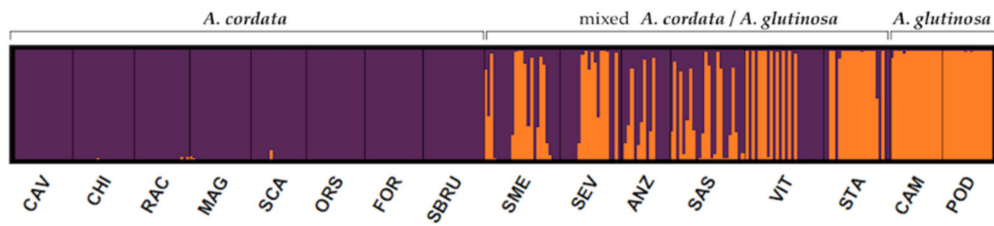
Figure 4. Genetic structure of 16 *Alnus* populations (8 pure *A. cordata*, 2 pure *A. glutinosa*, 6 mixed *A. cordata/A. glutinosa*) (core Group I = purple; core Group II = orange).

The analysis of molecular variance (AMOVA) was calculated separately for individuals of *A. cordata* and *A. glutinosa* (Table S3). In *A. cordata*, the partitioning of molecular variance was 89.86% within individuals, 5.86% among individuals within populations and 4.28% among populations. In *A. glutinosa*, the partitioning of variance was 77.5% within individuals, 12.98% among individuals within populations and 9.47% among populations.

The PCoA (Figure 5), performed on the complete set of individuals (*A. cordata*, *A. glutinosa* and hybrids), showed a clear separation between the pure species, with hybrid individuals falling in between the two, with a varying degree of kinship to the parental species. The hybrid individuals were genetically interspersed between the pure species and some of them overlapped with the *A. cordata* or *A. glutinosa* cluster, as an indication of "genetic admixture".
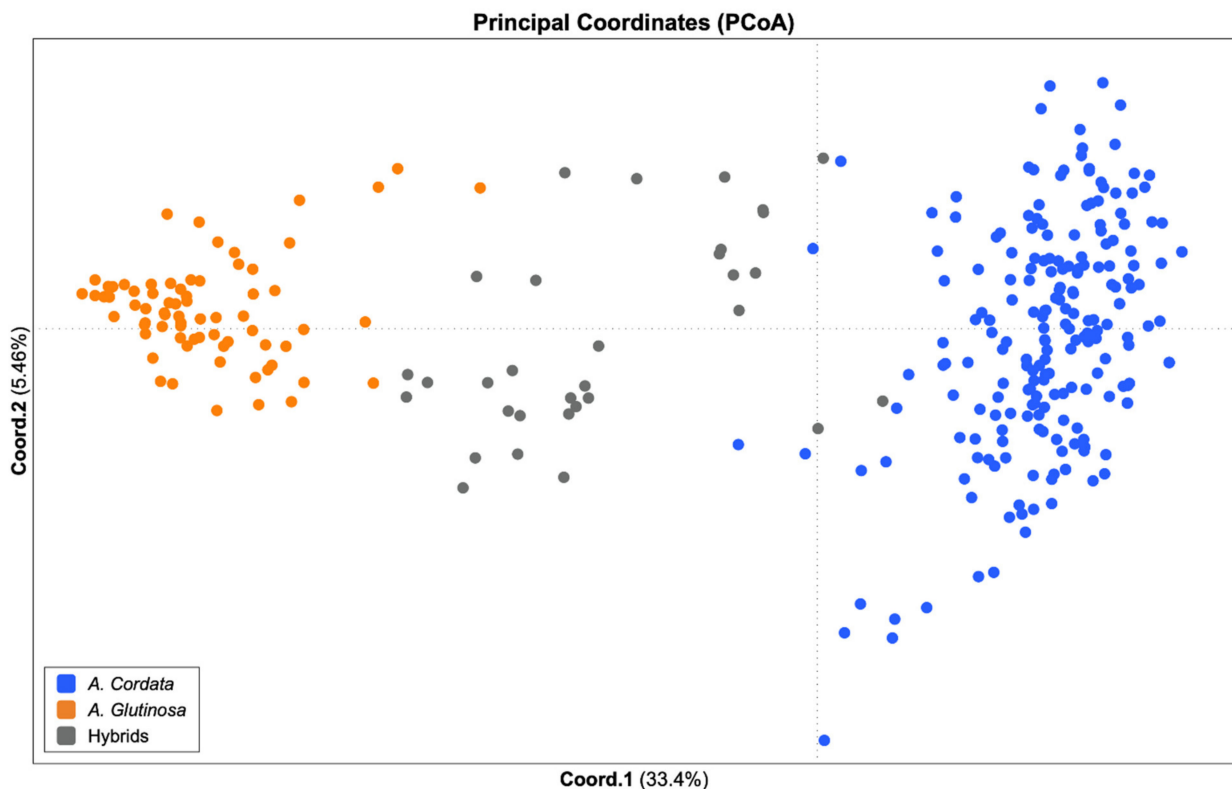

Figure 5. Principal Coordinate Analysis (PCoA) of pair-wise genetic distance based on the complete set of individuals of *A. cordata*, *A. glutinosa* and hybrids. Percentages of variation of the two axes are explained in the figure. Blue dots, *A. cordata* individuals; orange dots, *A. glutinosa* individuals; grey dots, hybrid individuals.

To better illustrate the relationships between the parental pure species and the hybrids, the average Nei's genetic distance was calculated between *A. cordata* and *A. glutinosa* and the group of hybrid individuals (Table S4). A greater genetic distance was found between

the hybrids and *A. glutinosa* (0.429) compared to that observed between the hybrids and *A. cordata* (0.247).

The results of UPGMA clustering (Figure 6) were in line with the average Nei's genetic distance (Table S4). The UPGMA tree highlighted three main clusters, the cluster of *A. cordata* populations, highly divergent from the cluster of *A. glutinosa* populations, and the cluster of mixed populations intermediate between the other two. Within the *A. cordata* cluster, subgroups can be identified, which correspond to the geographic position of the sampled populations. The populations with high presence of hybrid individuals were genetically closer to the pure populations of *A. cordata* then to *A. glutinosa*. The STA population, which was clustered close to *A. glutinosa* populations, confirmed this pattern, as it was composed mainly of *A. glutinosa* and a single hybrid individual was found in it.
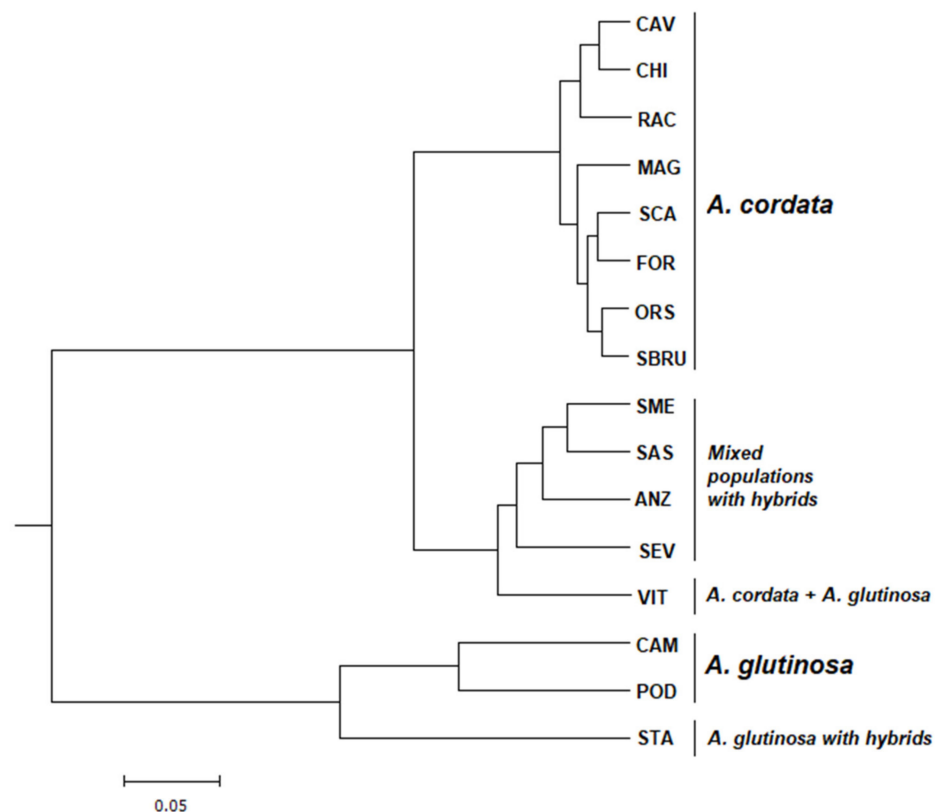

Figure 6. UPGMA cluster analysis based on Nei's genetic distances between pure and mixed populations of *A. cordata*, *A. glutinosa* and hybrids.

4. Discussion

Our study focused on *A. cordata*, an endemic species in southern Italy, we investigated its genetic variability and the introgression with *A. glutinosa,* a wide distributed species, which coexist in the same area.

Based on the genetic analysis, each individual was assigned to either pure *A. cordata*, pure *A. glutinosa* or different categories of hybrids (F$_1$, F$_2$, Backcrosses). We found the presence of hybrids in the mixed populations of *A. cordata*/*A. glutinosa* with a percentage from 9.1% (STA) to 50% (SAS). The majority of the hybrids identified in the mixed populations belonged to the F$_2$ category, with low frequency of backcrosses toward the parental species. Interestingly, F$_1$ hybrids were not detected in any of the mixed populations. We hypothesized that the F$_2$ individuals observed in our study could be the result of an "old" hybridization event; once rare F$_1$ hybrids are produced, they could have a good capacity to cross-breed with each other, producing F$_2$ individuals. The mixed population of VIT, represents a particular case, where the presence of both species was observed, but no hybrids were identified. This particular result indicates that hybridization between these

two species might be not common, as are the favorable conditions for its occurrence to arise. The variation of hybridization rate among the mixed populations suggests that natural crossing between the two species occurs at low and variable frequency, likely controlled by favorable local factors, which are unknown at present.

Other studies documented that crossing between two *Alnus* species might be difficult, even in the presence of a continuous mixed distribution where no natural barriers prevent gene flow between the two species [26,52,53]. A possible reason for this is the asynchronous flowering of the different species. The reproductive biology of *A. glutinosa* and *A. cordata* allows unidirectional crosses, with pollen of *A. glutinosa* fertilizing *A. cordata* flowers [31]. Experimental evidence supports the importance of climatic factors on hybridization success. Parfenov [53] studied the hybridization of *A. incana* with *A. glutinosa* in Belarus and noticed that the barrier to natural crossing laid in a six days shift in the flowering time between the two species. Such an impairment could be overcome in years with anomalous climate (e.g., with cold prolonged spring), when flowering time of the species had the chance to overlap. In another study of natural hybridization between *A. glutinosa* and *A. incana*, Banaev and Bazant [52] observed that hybrids occur very sporadically, even in a zone of continuous distribution of the species. They highlighted the increase in hybridization frequency in areas more affected by climate change. A similar hypothesis could explain the occurrence of hybrids observed in our case, as the study area is at the southern boundary of *A. glutinosa* geographic range and yearly climate fluctuations could rarely offset the flowering times of the two species and facilitate the hybridization. This hypothesis needs to be tested by further studies, but it raises the concern that climate change could affect interspecific hybridization in sympatric areas.

The structure and composition of the pure and mixed populations is further elucidated by STRUCTURE analysis, which highlighted two genetic clusters (K = 2), one represented by the *A. cordata* populations, and the other one by the *A. glutinosa* populations. A strong genetic homogeneity between individuals was evident within the pure populations of the two species, whereas in the mixed populations several hybrid individuals showed different levels of kinship to the main gene pools, sharing genetic background from the parental species.

The other main objective of this study was to evaluate the genetic diversity within and among the three main groups: *A. cordata*, *A. glutinosa* species and hybrids. All genetic diversity indices showed lower genetic variability level in *A. cordata* as compared to *A. glutinosa*, with hybrids showing the highest values in most indices. These results demonstrate that *A. cordata* is potentially more vulnerable in terms of genetic erosion. However, the observed level of interspecific hybridization and introgression did not reveal permeability between the two species, which could represent a threat for the genetic integrity of *A. cordata*. The analysis of genetic diversity of single populations highlighted that most of the pure *A. cordata* populations, except for the population ORS, showed a fixation index (F) close to zero, indicating that random mating occurred in these populations and no post-zygotic selection mechanisms favored inbreeding. Among the two *A. glutinosa* pure populations, the population CAM showed a significant positive F value (0.187). The geographic isolation of this population could explain its positive fixation index as a result of genetic drift. Despite their higher genetic diversity, all the mixed populations showed significant positive F values, corresponding to a deficiency of heterozygotes compared to the expected. This could be the result of the assortative mating between the two *Alnus* species with a small cohort of compatible individuals. Another point of great interest is the molecular signature of interspecific introgression as revealed by the differentiation pattern of the loci analyzed. The index Fst was highly variable across loci, with low value in the loci Alma 7 (0.062) and AG13 (0.088), and high values in the loci AG20 (0.515) and Alma1(0.320). This variation of Fst suggests a differential contribution of these loci and the associated genomic regions to the genetic structure and divergence between populations and species. The loci with high Fst are discriminant between populations, but also informative about the admixture rate between *A. cordata* and *A. glutinosa*. Actually, the allelic frequency of the

loci with high Fst were also highly divergent between populations and between species (Supplementary, Table S1). Reproductive isolation and introgression between species are largely controlled by "speciation" genes and the associated genome regions can be more or less porous to gene flow between species [54]. The analysis of Fst and allelic frequency in hybrid zones is a powerful approach to investigate the interspecific hybridization and the differential permeability of genomic regions to introgression [55]. In sympatric populations of *Juglans regia* L., *J. sigillata* Dode and *J. cathayensis* Dode, the analysis of genetic structure and Fst pattern by SSR markers have identified historical introgression phenomena and Fst outliers functionally linked to adaptive genes [56,57]. Genomic studies of sympatric populations of *Populus balsamifera* L., *P. angustifolia* E. James, and *P. trichocarpa* Torr. et A. Gray ex Hook. have discovered genomic clines of non-neutral introgression, with adaptive significance, especially at the geographic boundaries of species range [58]. Similarly, the variable Fst level observed in our study, at the southern margin of the *A. glutinosa* range, suggests that introgression between *A. cordata* and *A. glutinosa* might differentially affect loci and genome regions, with possible adaptive implications. This hypothesis needs to be elucidated in further studies of *Alnus* hybrid zones by genome-wide analyses.

5. Conclusions

We evaluated the genetic diversity of the endemic *A. cordata* populations in southern Italy and we reported the first molecular evidence of its natural hybridization with the widespread species *A. glutinosa*. The hybridization between the two species naturally occurs in sympatric areas, but the phenomenon varies in different ecological sites. The very low frequency of backcross individuals suggests a limited capacity of hybrid individuals to cross back with the parent species, reducing the risk of genetic pollution of *A. cordata*. Therefore, the hybrid individuals seem not to have the potential to wipe out the parental species and the genetic integrity of the endemic *A. cordata* would not be endangered. The results support the hypothesis that the hybridization occurs under favorable, but rare circumstances. The factors that actually favor this phenomenon are not investigated in this study, but reasonable hypotheses are related to particular climatic conditions that allowed the species to cross and produce F_1 hybrids in an initial event. In this scenario, climate change could affect the flowering time of the two species, and possibly facilitate interspecific hybridization. Further studies could clarify the conditions under which hybridization between the two species has the potential to occur. In conclusion, this work might provide meaningful knowledge for the conservation and management of the endemic species *A. cordata*, which represents a valuable resource of biodiversity in the Mediterranean ecosystems.

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