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Genetic diversity and genetic structure of black alder (*Alnus glutinosa* [L.] Gaertn) in the Belgium-Luxembourg-France cross-border area

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Abstract Due to its beneficial effects on river ecosystems, black alder (*Alnus glutinosa*) is one of the tree species selected for planting on riverbanks in the cross-border area encompassing Wallonia in Belgium, Lorraine in France, and Luxembourg. The preservation of this species, however, is threatened by an invasive pathogen that particularly targets and kills young alder individuals. The objectives of this study were to characterize the genetic diversity and the genetic structure of *A. glutinosa* at this local level with the aim of assisting the conservation and replanting strategies and to determine if a germplasm collection comprising individuals from the same cross-border area captures the diversity present in the region. Nuclear simple sequence repeat (SSR) and chloroplastic DNA (cpDNA) markers were used to analyze four local wild populations and the germplasm collection which is representative of two river catchments and six legal provenance regions. Three populations distant from the studied area were also included. A panel of 14 nuclear SSR loci revealed high allelic diversity and very low differentiation among wild populations (mean $F_{ST} = 0.014$). The germplasm collection displayed a range of alleles that were representative of the different populations, and no significant differentiation between the germplasm collection and the local wild populations was observed, making this collection, as far as allelic diversity is concerned, suitable for providing trees for riverbank replanting programs. Using SSR markers, various statistical approaches consistently indicated the lack of a significant geographical structure at the level of the river catchments or provenance regions. In contrast, two cpDNA haplotypes were detected and displayed a cross-border geographically structured distribution that could be taken into account in defining new cross-border provenance regions.

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Introduction

The protection and management of natural resources has become a major challenge for land managers and conservationists. In order to address the degradation of riparian ecosystems, and in response to the need to comply with European directives on protecting the ecological status of rivers, a management policy based on replanting riverbanks is being implemented in the cross-border area that includes Wallonia in Belgium, Lorraine in France, and Luxembourg. This replanting program involves maintaining and planting trees of various species that are beneficial to river ecosystems,

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including black alder (*Alnus glutinosa* [L.] Gaertn.), one of the major species of riparian ecosystems in Europe. *Alnus glutinosa* is attracting increasing interest because of its beneficial effect on riparian ecosystems (Claessens et al. 2010). It contributes to water filtration and purification (Peterjohn and Correll 1984) and its root system has a positive effect on riverbank stabilization (Bibalani et al. 2008).

Alnus glutinosa is a threatened species in the region. Since the early 1990s, it has been endangered by the hybrid oomycete *Phytophthora × alni*, leading to dieback in riparian ecosystems (Husson et al. 2015). This pathogen is the result of interspecific hybridization between two taxa, *P. uniformis* and *P. × multiformis*. Alder in the Rhine-Meuse basin in the Wallonia-Lorraine-Luxembourg region is particularly threatened by this emerging disease (Thoirain et al. 2007), although climatic conditions have, to some extent, greatly limited its severity (Aguayo et al. 2014). Young seedlings, however, are particularly affected by the disease and die quickly after infection, threatening the long-term survival of black alder in the area (Elegbede et al. 2010). In addition, *A. glutinosa* has exacting ecological requirements due to its intolerance of shade and its ecological preference for wet sites. Many sites that would have been suitable for black alder in the past have been converted to agriculture, leading to a scattered distribution of small discrete populations. With these threats in mind, there is a need to produce trees for replanting. The use of autochthonous material is currently recommended as growth problems have been recorded in Germany in *A. glutinosa* plantations based on material from distant sources which were probably poorly adapted to the local conditions (Kajba and Gracan 2003).

Within this context, a germplasm collection of healthy *A. glutinosa* trees growing along watercourses in Wallonia, Luxembourg, and Lorraine was established (Druart et al. 2013) in order to preserve the local diversity of the species and to provide trees for the replanting program. This material could be used further to produce rooted cuttings and/or establish a seed orchard, depending on the results of our study and on phenotypic observations. The sampling area chosen ensured that this collection was representative of the cross-border region and of the distribution of *A. glutinosa* in the two main river catchments (Meuse and Rhine) and the different provenance regions. These provenance regions were defined individually by the Walloon (Direction des Ressources Forestières 2011) and French (Rousselet et al. 2003) authorities according to the European Directive (1999/105/EC) regulating the marketing of forest materials. They apply to reforestation, but are not mandatory for the replanting of riverbanks.

When planning a replanting program, consideration should be given to any genetic structure at a local scale and differentiation at a river catchment, provenance region, or national scale. An analysis of the genetic diversity at this local scale will provide valuable information for river managers.

At the European scale, King and Ferris (1998) showed a highly structured geographical distribution of chloroplastic (cp) DNA haplotypes and hypothesized that, after the last glacial period, black alder colonized most of northern and central Europe from a refuge in the Carpathian Mountains. This model has recently been challenged by a continent-wide population genetic study (Havrdová et al. 2015), suggesting that multiple refugia served as sources for the postglacial colonization of Europe by *A. glutinosa*, central Europe being an area of genetic admixture between western and eastern lineages originating from the northern part of the Iberian Peninsula and the Carpathians, respectively. Using SSR markers, Lepais et al. (2013) showed a strong distinctiveness of Moroccan populations of *A. glutinosa* in the rear-edge distribution margins of the species. Recently, two studies using SSR markers have shown high levels of genetic diversity and very little differentiation between Irish *A. glutinosa* populations (Beatty et al. 2015; Cubry et al. 2015). Similarly, a recent study based on population genomics and conventional provenance trials conducted mainly in Flanders in Belgium showed little differentiation in adaptative traits among populations (De Kort et al. 2014).

The aim of our work was to study the genetic diversity and structure of *A. glutinosa* in the Wallonia-Lorraine-Luxembourg cross-border area involved in the riverbank replanting program. Therefore, nuclear SSR and chloroplastic DNA (cpDNA) markers were used to: (i) compare four local wild populations representative of the three river catchments and provenance regions; (ii) compare these populations with more distant European populations; (iii) assess whether the existing germplasm collection captured the genetic diversity of the cross-border area; and (iv) determine whether neutral genetic differentiation occurred among provenance regions and/or river catchments based on an analysis of both the wild populations and the germplasm collection together.

Materials and methods

Study species

Black alder (*Alnus glutinosa* [L.] Gaertn., $2n = 28$) is a deciduous tree belonging to the Betulaceae family (Betuloideae subfamily). It is a wind-pollinated self-incompatible species (Steiner and Gregorius 1999). Seeds can be dispersed up to 30 m by wind (McVean 1953), though dispersal is mainly by water in rivers. As a pioneer species with fast initial growth, it can rapidly colonize bare ground. Usually found along rivers and streams, alder populations tend to be linear in shape, extending downstream along riverbanks. The species is found in most of Europe and its natural range extends to North Africa and Asia Minor.

Plant material

The region under study encompassed the cross-border area of Wallonia in Belgium, Lorraine in France, and Luxembourg. The sampling strategy included material from three sources. The entire sample consisted of four populations from the cross-border area, three more distant populations from Poland, Denmark, and Sweden and the germplasm collection comprising individuals from the same cross-border area as above. This material was classified according to the country of origin, river catchment, and provenance region in which it occurred (Table 1). In total, 305 trees were genotyped (see Additional File 1).

The four populations sampled in the cross-border area (Fig. 1) were woodland populations considered to be natural *A. glutinosa* stands, preserved from plantation and are representative of the river catchments of the Scheldt (one site), the Meuse (one site), and the Rhine (two sites). Three populations from Poland, Denmark, and Sweden provided external references to the area of interest (Fig. 1b). Plant material from Sweden was sampled in a region corresponding to the northern limit of the geographical distribution area of *A. glutinosa*. In each of the seven populations, sampling was conducted over a few hundred meters with a minimum distance of 15 m between trees.

The germplasm collection was established from cuttings of wild and healthy *A. glutinosa* trees growing along rivers spanning the whole cross-border area (Druart et al. 2013) (Fig. 1a). Each genotype in the collection is represented by a unique rooted cutting taken from each wild tree sampled. The cuttings were collected at 78 sampling sites (see Additional File 1).

Leaves from each tree were collected. They were frozen in liquid nitrogen and stored at −80 °C (cross-border area) or dried and stored at room temperature (Poland, Denmark, Sweden).

DNA extraction, PCR amplification, and electrophoresis

Genomic DNA was extracted from 200 mg of leaf tissue from each of the 305 genotypes using the DNeasy Plant Mini Kit (Qiagen). The quantity and quality of the DNA were checked on an agarose gel. Individuals were genotyped for 14 nuclear SSRs (Table 2). Some of these markers were developed in *Corylus avellana* (Bassil et al. 2005; Boccacci et al. 2005) or in *Betula pendula* (Kulju et al. 2004). Their cross-transferability in *A. glutinosa* has been demonstrated by Zhuk et al. (2008) and Mingeot et al. (2010). PCR reactions were performed in a total reaction volume of 20 μl containing about 20 ng of genomic DNA, 1× PCR buffer, 0.2 mM of each dNTP, 1.5 mM $MgCl_2$, 1 μg/μl BSA, 0.25 μM of each primer, and 0.5 U Taq DNA polymerase (Fermentas). The initial denaturation step at 94 °C for 4 min was followed by 30 cycles of 1 min at 94 °C, 1 min 15 s at the specific annealing temperature of each primer pair (Table 2) and 2 min 30 s at 72 °C. The PCR ended with a final elongation step at 72 °C for 10 min. The electrophoresis was performed in 6.5 % denaturing polyacrylamide gel using a Li-Cor global edition IR2 DNA sequencer (Westburg). Allele scoring was done using Gene ImagIR software, v 4.03 (Westburg).

Chloroplast fingerprinting was performed on the 235 samples from the cross-border area using the universal primers pairs trnC[tRNA-Cys (GCA)]-trnD [tRNA-Asp (GUC)] and

Table 1 Location of the sampled populations and original locations of material in the germplasm collection

Site name		Country	Longitude	Latitude	Sample size	River catchment			Provenance region					
						Scheldt	Meuse	Rhine	1	2	3	4	5	6
Cross-border area	Wild populations													
	Fourneau St Michel	Belgium	50.07	5.38	25	–	25	–	–	–	–	25	–	–
	Schoenberg	Belgium	50.29	6.27	25	–	–	25	-	-	-	25	–	–
	Ligne	Belgium	50.61	3.71	15	15	–	–	15	–	–	–	–	–
	Gosselming	France	48.81	7.01	21	–	–	21	–	–	–	–	–	21
	Germplasm collection	Belgium	–	–	84	2	76	6	5	1	28	45	5	0
		France	–	–	37		3	34						37
		Luxemburg	–	–	28			28						
Total					235	17	104	114	20	1	28	95	5	58
Outside cross-border area	Blizin	Poland	51.07	20.73	26									
	Esrum	Denmark	55.98	12.38	20									
	Umeå	Sweden	63.77	20.44	24									
Total					305									

Provenance regions 1 to 5 are in Belgium, provenance region 6 is in France. *1*: Brabant, *2*: Hesbaye, *3*: Bas plateaux mosans, *4*: Ardenne, *5*: Gaume, *6*: Nord-Est et montagnes

Fig. 1 Geographic location of the black alder (*Alnus glutinosa*) sampling sites. **a** Map of the cross-border area. The locations of the trees constituting the germplasm collection are shown by small circles; the populations sampled are shown by large circles. Three watersheds are represented: Meuse (*grey*), Rhine (*black*), and Scheldt (*white*). Samples from the same provenance region are surrounded by a *green line*. **b** Location of the distant populations sampled
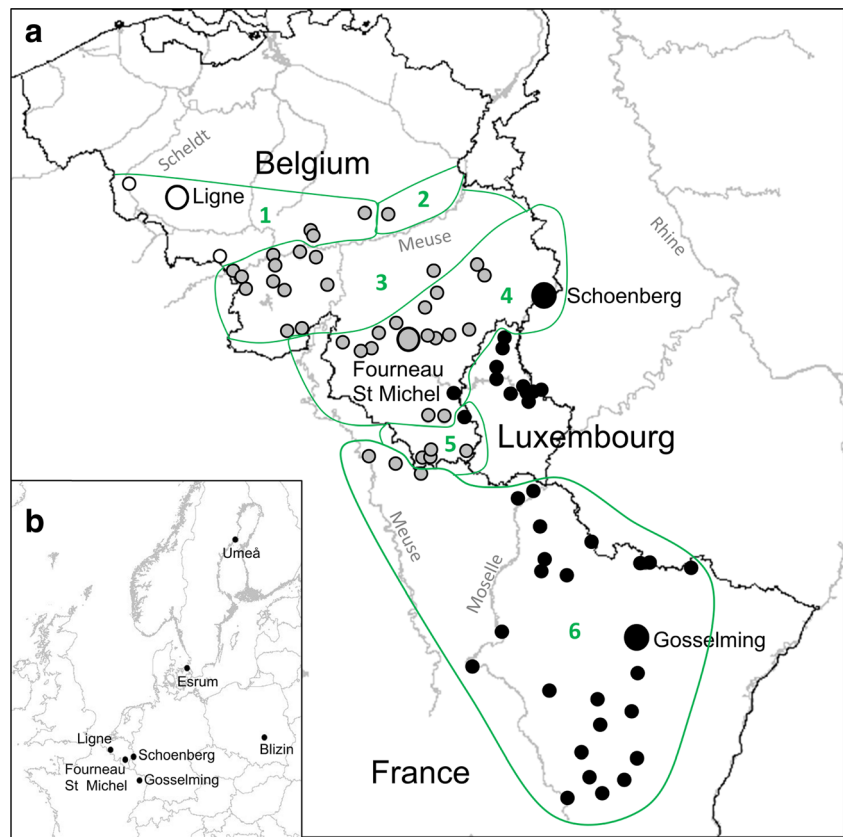
trnK1 [tRNA-Lys (UUU) 3′exon]- trnK2 [tRNA-Lys (UUU) 5′exon] of Demesure et al. (1995). PCR was carried out in a total volume of 25 μl consisting of 20 ng of genomic DNA, 1× PCR buffer, 1 μg/μl BSA, 0.2 mM of each dNTP, 2 mM MgCl$_2$, 0.25 μM of each primer, and 0.5 U Taq DNA polymerase (Fermentas). The PCR program recommended by King and Ferris (1998) was used, involving an initial denaturation step at 94 °C for 5 min, followed by 30 cycles of 30 s at 94 °C, 30 s at 58 °C (trnC-trnD), or 53 °C (trnK^1-trnK^2) and 3 min at 72 °C, and a final elongation step at 72 °C for

Table 2 Characteristics and diversity information parameters at 14 SSR loci used on 235 *Alnus glutinosa* genotypes from the cross-border area

SSR	Reference	T (°C)	Na	Ne	Ho	He	PIC
CAC-A105	Bassil et al. (2005)	50	32	14.8	0.80	0.93	0.927
CAC-B101	Bassil et al. (2005)	60	12	3.8	0.64	0.74	0.701
CAC-B029b	Bassil et al. (2005)	50	8	2.6	0.57	0.61	0.573
CAC-B109	Bassil et al. (2005)	50	2	1.3	0.20	0.23	0.212
CAC-B113	Bassil et al. (2005)	50	12	4.4	0.86	0.77	0.739
CaT-B106	Boccacci et al. (2005)	50	2	1.2	0.18	0.16	0.15
CaT-B507	Boccacci et al. (2005)	55	5	1.4	0.29	0.28	0.264
CAC-C118	Bassil et al. (2005)	55	6	2.3	0.57	0.57	0.533
Ag164I	Mingeot et al. (2010)	58	36	7.8	0.68	0.87	0.862
L2.7	Kulju et al. (2004)	58	32	15.4	0.86	0.93	0.931
L3.1	Kulju et al. (2004)	50	14	5.3	0.67	0.81	0.789
L5.4	Kulju et al. (2004)	57	6	2.5	0.57	0.61	0.534
L5.5	Kulju et al. (2004)	52	8	4.0	0.48	0.75	0.707
L7.8	Kulju et al. (2004)	57	14	5.2	0.78	0.81	0.791
Mean			14	5.2	0.588	0.651	0.626

Optimal annealing temperature (T[°C]), number of alleles (*Na*), number of effective alleles (*Ne*), observed (Ho), and expected (He) heterozygosity, polymorphism information content (PIC)

10 min. The PCR product (10 μl) was restricted overnight at 37 °C with 10 U of the enzymes *Hha*I and *Alu*I (trnC-trnD) or *Hae*III and *Hinf*I (trnK^1-trnK^2) in a total volume of 32 μl. Electrophoresis of the restricted PCR products was performed in 5 % non-denaturing polyacrylamide gel stained with silver nitrate for trnC-trnD and in 1.6 % agarose gel for trnK^1-trnK^2. The haplotypes were scored following the notation used by King and Ferris (1998).

Data analysis

For each nuclear SSR locus, the total number of alleles (Na), the number of effective alleles (Ne), the observed heterozygosity (Ho), and the expected heterozygosity (He) were calculated using GenAlEx software, v 6.2 (Peakall and Smouse 2006). The polymorphism information content (PIC) was evaluated as described by Botstein et al. (1980) using PICcalc (Nagy et al. 2012). For each population across loci and for the germplasm collection, the Na, the mean number of alleles per locus (Nam) and the number of private alleles (Ap) were calculated using GenAlEx, v 6.2 (Peakall and Smouse 2006). Allelic richness (number of alleles per locus, independent of sample size) (Â) was calculated using Fstat, v 2.9.3.2 (Goudet 1995), using the rarefaction method. Rarefaction was done based on a minimum sample size of 13, which corresponded to the smallest number of individuals successfully genotyped for all loci in the population from Ligne. Using Genepop, web v 4.2 (Rousset 2008), observed heterozygosity (Ho), expected heterozygosity (He), and inbreeding coefficient (F_{IS}) were calculated. Pairwise F_{ST} values between populations were estimated using GenAlEx, v 6.2. The ratio of pollen flow to seed flow was calculated on the basis of the four populations from the cross-border area using the Ennos formula (Ennos 1994): (pollen flow/seed flow) $= ((1/F_{ST(b)} - 1) - 2(1/F_{ST(m)} - 1))/(1/F_{ST(m)} - 1)$, where $F_{ST(b)}$ and $F_{ST(m)}$ are the levels of genetic differentiation among the populations based on biparentally (nuclear SSR) and maternally (cpDNA) inherited markers, respectively. The presence of null alleles was checked using Micro-Checker, v 2.2.3 (Oosterhout et al. 2004).

The model-based clustering method in STRUCTURE software, v 2.3 (Pritchard et al. 2000), was applied to the SSR data to infer the genetic structure in the dataset. The method was applied to the data of the four populations from the cross-border area (86 samples), to the data of all the samples from the cross-border area (germplasm collection and four populations, 235 samples) and adding the data from the more distant populations (305 samples). In each case, 10 independent runs were conducted for each value of K ranging from 1 to 20. Each run consisted of 100,000 burn-in steps, followed by 100,000 Monte Carlo Markov Chain (MCMC) iterations. We used the admixture model and assumed independent allele frequencies. In order to determine the optimal K value, the ΔK statistic developed by Evanno et al. (2005) was calculated

using STRUCTURE HARVESTER software (Earl and vonHoldt 2012). CLUMPP software (Jakobsson and Rosenberg 2007) was used to obtain the mean individual Q-matrix. An analysis of molecular variance (AMOVA) was performed for both cpDNA haplotypes and nuclear SSR markers using Arlequin software, v 2.0 (Schneider et al. 2000), to investigate divergence among populations, watersheds, and provenance regions. The analysis was performed independently for each of these levels because of the different number of genotypes tested for each level (see Table 1). Because of the limited number of samples, provenance regions 2 and 5 were not included.

Results

Overall nuclear microsatellite diversity in the cross-border area

Alnus glutinosa showed a high level of genetic diversity in the cross-border area, with an average of 14 alleles per locus for the 235 genotypes tested (Table 2). The 14 SSR markers used allowed 232 genotypes to be distinguished: 230 trees had a unique allelic combination, whereas the five remaining trees, originating from the germplasm collection, were divided into two groups (two and three trees) sharing the same genotype. As they were collected along the same rivers, the trees with shared genotypes could be clones originating from the vegetative regeneration of sprouts by root suckers or by the rooting of lateral branches.

In total, the 14 SSR markers detected 189 alleles, including 58 rare alleles (frequency <1 %). The microsatellite loci displayed a wide range of PIC values, with an average of 0.626. For all loci, Ne was much less than Na, indicating significant differences in allele frequencies and the presence of rare alleles. The mean He (0.65) slightly exceeded the mean Ho (0.59), indicating a deficit in heterozygotes, and He was higher than Ho at 11 of the 14 loci.

Genetic analysis at the local population level

The cpDNA trnC-trnD and trnK^1-trnK^2 markers used allowed us to distinguish the two haplotypes (F and G) described by King and Ferris (1998). The two haplotypes were unevenly represented among the populations: the Fourneau Saint Michel population displayed haplotype F only, the Gosselming population haplotype G only, the Ligne population 69 % haplotype F and the Schoenberg population 88 % haplotype F (Table 3).

The 14 nuclear SSR markers used on the four populations sampled in the cross-border area were polymorphic at all the sampling sites and the total number of alleles per population ranged from 88 to 111, with an average of 7.34

Table 3 Genetic diversity within *Alnus glutinosa* populations

	N	SSR markers						cpDNA haplotypes	
		Nat	Nam	Â	Ho	He	F_{IS}	F	G
Border area									
Fourneau St Michel	25	99	7.64	6.12	0.61	0.62	0.011	25	0
Schoenberg	25	111	7.93	6.36	0.63	0.65	0.032	22	3
Ligne	15	88	6.29	6.03	0.57	0.65	0.115***	9	4
Gosselming	21	111	7.50	6.40	0.66	0.66	0.002	0	21
Mean		102.3	7.34	6.226	0.62	0.64	0.040		
Outside border area									
Blizin	26	115	8.21	6.53	0.59	0.63	0.064*		
Esrum	20	103	7.36	6.49	0.63	0.66	0.055*		
Umeå	24	80	5.71	4.99	0.59	0.60	0.017		

Sample size (*N*), total number of alleles (Nat), mean number of alleles per locus (Nam), allelic richness (Â), observed heterozygosity (Ho), expected heterozygosity (He), inbreeding coefficient (F_{IS})

*Significant at the 5 % level, ***significant at the 0.1 % level

alleles per locus and per population (Table 3). The allelic richness (Â) did not vary significantly among populations, with an average of 6.23. He consistently exceeded Ho for all populations. The F_{IS} was positive for all sites (mean value = 0.040), reflecting the deficit in heterozygotes, and it varied greatly depending on the population. The F_{IS} value was highly significant ($P < 0.001$) for Ligne, which deviated significantly from the Hardy-Weinberg equilibrium. Three of the six pairwise differentiation tests among populations from the cross-border area were significant at $P < 0.01$ (Table 4). The three other pairwise comparisons all concerned the Ligne population. In all cases, however, the magnitude of genetic differentiation among populations was very low, with a mean F_{ST} of 0.014.

Combining the F_{ST} values of the four populations from the cross-border area for the nuclear SSR and cpDNA haplotypes enabled us to show that the level of pollen flow was 194-fold higher than the seed flow.

Comparison between the local cross-border area and the distant populations

The 14 nuclear SSR markers were also used on the three distant populations used as external references (Tables 3 and 4). The populations from Denmark and Poland (Esrum and Blizin) exhibited mean numbers of alleles and allelic richness similar to the populations Fourneau St Michel, Schoenberg, and Gosselming from the cross-border area. In contrast, the population from Umeå in Sweden exhibited significantly lower allelic richness (4.99). It was also the most genetically distinct, with highly significant ($P < 0.001$) pairwise F_{ST} values ranging from 0.032 to 0.067, whereas the pairwise comparisons among the populations from the cross-border area and from Denmark and Poland were significant, but displayed low F_{ST} values. The comparisons between the population from Ligne and each of the other populations were not significant, except for Umeå.

Table 4   Matrix of pairwise F_{ST} values among seven *Alnus glutinosa* populations

N°	Population	1	2	3	4	5	6
1	Fourneau St Michel						
2	Schoenberg	0.021**					
3	Ligne	0.015*	0.010*				
4	Gosselming	0.017**	0.013**	0.006			
5	Blizin	0.020**	0.014**	0.003	0.014**		
6	Esrum	0.019**	0.021**	0.005	0.019***	0.015**	
7	Umeå	0.067***	0.045***	0.032**	0.045***	0.040***	0.038***

*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. Pairwise comparisons involving the distant populations are shaded in *grey*

A total of 196 alleles were detected in the seven populations, of which 32 were private alleles. The Ligne and Esrum populations were the only ones with private alleles with frequencies of ≥5 % (two and three alleles, respectively). Evidence for the presence of null alleles was detected only for loci CAC-B101 in Ligne, L27 in Umeå, and Ag164I in Umeå, Ligne, and Esrum.

Genetic analysis of the germplasm collection

The cpDNA markers were used on the 149 samples of the germplasm collection. Haplotype F was the most frequent, with 68 % of the trees displaying this haplotype. The two haplotypes were not uniformly distributed across the area (Fig. 2). The distribution of cpDNA haplotypes in the collection corroborated that of the haplotypes in the corresponding populations.

The 14 nuclear SSR markers used on the germplasm collection displayed 176 alleles and the allelic richness of the germplasm collection (6.75) slightly exceeded the mean allelic richness of the populations from the same area. Only eight alleles from the four populations of the cross-border area were not represented in the collection, and all eight of them were infrequent (frequency < 5 %). In contrast, the germplasm collection displayed 26 private alleles that were not represented

in any population; these 26 alleles were rare (frequency < 5 %). No significant differentiation was detected between the germplasm collection and the populations from the cross-border area ($F_{ST} = 0.005$). The germplasm collection therefore appeared to capture the allelic diversity of the natural local populations.

Partitioning the samples of the germplasm collection among the corresponding river catchments or provenance regions (only classes with at least 20 trees were considered) did not show any significant difference in Ho, He, or Â with regard to these subdivisions (Table 5).

Genetic differentiation and geographic structure in the cross-border area

The model-based clustering method in STRUCTURE software was applied to the SSR data to infer the genetic structure in the cross-border area. The software failed to detect any structure, either among the four populations or taking account of all the samples of the area (germplasm collection and populations together). Using the LOCPRIOR option to assist the clustering, with either the river catchment or provenance region as prior information, did not improve the results. When the SSR results of the distant populations were added, a maximum

Fig. 2 Distribution of chloroplast haplotypes in *Alnus glutinosa* in the cross-border area. The sizes of the pie charts vary according to sample size
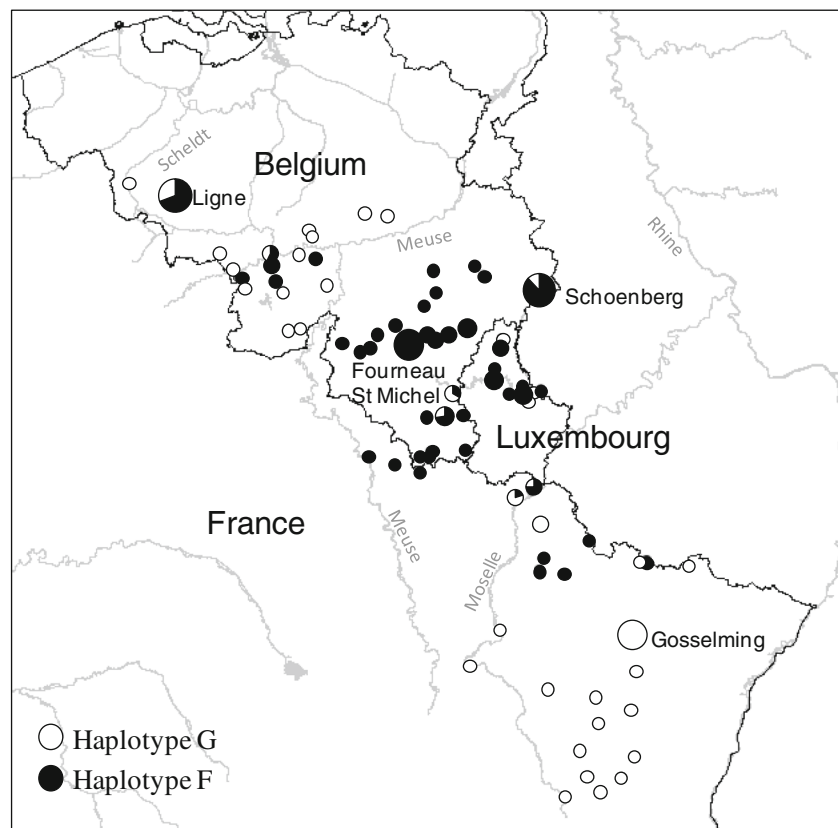
Table 5 Genetic diversity within the *Alnus glutinosa* germplasm collection

	N	SSR markers					cp DNA haplotypes	
		Nat	Nam	Â	Ho	He	%F	%G
Germplasm	149	176	12.571	6.75	0.56	0.65	68	32
Meuse	79	157	11.214	6.80	0.55	0.64	78	22
Rhine	68	149	10.643	6.58	0.57	0.64	57	43
Region 3	28	120	8.571	6.73	0.54	0.64	64	36
Region 4	45	138	9.857	6.71	0.55	0.63	89	11
Region 6	37	125	8.929	6.43	0.57	0.64	27	73

Sample size (*N*), total number of alleles (Nat), mean number of alleles per locus (Nam), allelic richness (Â), observed heterozygosity (Ho), expected heterozygosity (He)

LnP(D) value was found at $K = 6$, corresponding to a weak peak in the curve of ΔK statistics (Evanno et al. 2005). The mean individual probability of assignment to the six clusters was very low ($q = 0.47$). Interestingly, the 24 trees from the Umeå population could be assigned to the same cluster (Fig. 3, green cluster), with a mean q value of 0.77 for this population.

The AMOVA results are given in Table 6. The use of nuclear SSR markers showed that there was a very low percentage of variation among populations (1.5 %), river catchments (0.5 %) and provenance regions (0.4 %), although these values were significant between populations and watersheds ($P < 0.001$ and $P < 0.01$, respectively). In contrast, the cpDNA haplotypes showed a high percentage of differentiation between river catchments (14 %) and provenance regions (48 %) and very high differentiation among populations (75 %). With both marker systems, therefore, the differentiation was higher between populations than between river catchments and provenance regions.

An additional AMOVA analysis with the SSR data was conducted to compare both cpDNA haplotypes to each other and did not show any significant difference (0.5 % of the variance explained, data not shown).

Discussion

Genetic diversity based on cpDNA variation

In a survey of cpDNA diversity on a European scale, King and Ferris (1998) reported a high degree of structuring of 13 haplotypes, two of which occurred in northern and central Europe (i.e., covering the area of our study). However, Belgium and northern France were represented by only six trees in their study. In contrast, Havrdová et al. (2015), using another marker system of cpDNA, revealed the existence of 43 haplotypes on the European scale, of which only one covered the area of our study. Using the same marker system as King and Ferris (1998), we found the same two haplotypes, F and G, in the area concerned. Our results, based on a large sample of 235 trees, provided more insight into the distribution of the haplotypes in the Wallonia-Lorraine-Luxembourg cross-border area. We showed that haplotypes F and G displayed a geographically structured distribution, even at such a small scale. In the area between the Meuse and Moselle rivers (Ardennes Massif), haplotype F was predominant (91 %); in the area north of the Meuse, there was a mixture of both haplotypes (53 % haplotype F); and east of the Moselle, haplotype G was predominant (79 %). Due to the geographic region covered by the study, however, our results do not enable us to hypothesize about the postglacial migration routes of the species.

Genetic diversity based on nuclear SSRs

The panel of 14 SSR loci revealed high allelic diversity in the cross-border area, with an average of 14 alleles per locus. The average PIC (0.626) and genetic diversity (He = 0.65) values were similar to those reported by Lepais and Bacles (2011) for Scottish *A. glutinosa* samples (average PIC = 0.68; average He = 0.69) and the He value was consistent with mean values from microsatellite data obtained for Irish, Scottish, and French populations (Cubry et al. 2015; He = 0.64) and for Northern Irish populations (Beatty et al. 2015; He = 0.66). These values were consistent with the mean values from
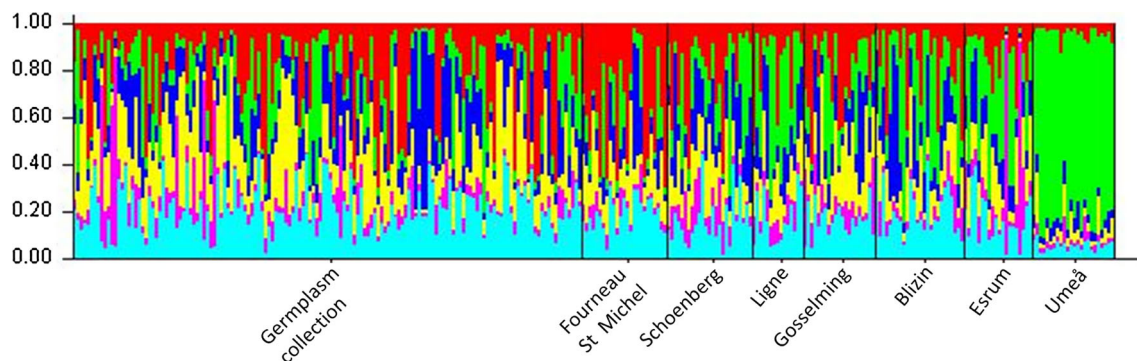

Fig. 3 Inference of population structure in black alder (*Alnus glutinosa*) using STRUCTURE software, v 2.3, STRUCTURE clustering results for $K = 6$

Table 6 Differentiation (F_{ST}) among populations, river catchments, and provenance regions estimated using AMOVA analysis

	Nuclear SSR	Cp haplotypes
Partitioning among four populations ($n = 86$)		
Among populations	0.015**	0.750**
Within populations	0.985	0.250
Partitioning among three river catchments ($n = 235$)		
Among watersheds	0.005*	0.140**
Within watersheds	0.995	0.860
Partitioning among four provenance regions ($n = 202$)		
Among provenance regions	0.004	0.477**
Within provenance regions	0.996	0.523

$*P < 0.01$, $**P < 0.001$

microsatellite data obtained from a bibliographic compilation by Nybom (2004) for wind and/or water seed-dispersal species (He = 0.61). However, all these studies were realized using different panels of SSR loci.

Genetic diversity at the local population level in the cross-border area

Each population in the cross-border area showed high allelic diversity. The four wild populations from the cross-border area displayed a similar allelic richness that was consistently lower than the mean number of alleles per locus, reflecting large frequency differences among alleles and a high number of rare alleles. A fairly high number of private alleles was detected among the different populations, but this likely reflected the abundance of rare alleles (low frequencies) rather than local differentiation.

The F_{IS} was positive for all sites (mean value = 0.040). Similar F_{IS} values were reported from Northern Irish scattered populations (Beatty et al. 2015). The F_{IS} value for Ligne ($F_{IS} = 0.115$) was highly significant ($P < 0.001$), although the allelic richness of this population did not differ significantly from that of the other populations.

This result should be viewed with caution because it might have arisen from the limited number of samples (15 trees), combined with the use of multi-allelic markers. This could also be interpreted, however, as the effect of a small population on the F_{IS} coefficient. The small number of trees sampled from this site was a consequence of the small size of this population, because it was not possible to find a higher number of non-adjacent trees. Given the environmental requirements of *A. glutinosa*, such scattered small populations are common in agricultural regions. Lowe et al. (2005) demonstrated that a decrease in population size would lead to an immediate increase in the F_{IS} (due to selfing or mating between relatives), but the impact on genetic diversity and allelic distribution would only become apparent after several generations.

The mean genetic differentiation among populations was low, with an average F_{ST} value of 0.014. A similar low level of differentiation was found among Northern Irish (Beatty et al. 2015; $\Phi_{ST} = 0.0195$) and Irish populations (Cubry et al. 2015).

Combining our cpDNA and nuclear marker results, we estimated that the relative rate of pollen flow to seed flow was 194-fold for *A. glutinosa*. This exceeds the estimate given by King and Ferris (1998) (pollen flow/seed flow = 23), but this figure was based on combining cp results (King and Ferris 1998) and isozymes results (Prat et al. 1992) obtained from different populations. The present study provides an estimation based on either maternally or biparentally inherited markers used on the same samples. This high pollen/seed flow ratio could be explained by the reproductive and dispersal patterns of this species. High rates of interpopulation pollen dispersal would be expected because *A. glutinosa* is a wind-pollinated species. Seed dispersal is expected to be low. Indeed, it occurs mainly via flowing water rather than via wind, and is therefore restricted to a distance of about 30 m from the parents (McVean 1953). Despite the high potential for seed dispersal over long distances by water, *A. glutinosa* dispersal is limited by its intolerance to shade, which restricts seed germination and survival in areas already colonized by trees. The pollen:seed dispersal ratio estimated for *A. glutinosa* is similar to that obtained for oak (196), also a wind-pollinated tree, whose acorns are dispersed by birds and rodents, and it is considerably higher than the ratios of various *Pinus* species (20 to 68, depending on the species) that have wind-dispersed pollen and seeds (Ennos 1994).

Comparison with distant populations

The differentiation of the cross-border populations from the Danish and Polish populations was low (F_{ST} values ranging from 0.003 to 0.021). These results are compatible with those of Cubry et al. (2015), who did not find any significant differentiation among populations from Scotland and France and wild Irish populations. Therefore a lack of genetic structure has been observed across large geographic scales in several studies of *A. glutinosa*.

In contrast, the Umeå population displayed highly significant differentiation from all the other populations and had the lowest allelic richness. It showed no sign of inbreeding ($F_{IS} = 0.017$), however. The sampling site at Umeå (latitude 63.77°N) was located at the northern limit of *A. glutinosa* distribution. The decrease in within-population variation combined with the increase in population differentiation and no significant inbreeding in Nordic marginal regions has been described for the European hazelnut (*Corylus avellana*), also a member of the Betulaceae family (Persson et al. 2004).

Analysis of the germplasm collection

The germplasm collection was established with the intended aim of being representative of the distribution of *A. glutinosa*

in the cross-border area. It was based on cuttings taken from healthy riverbanks trees from across the whole area, including three river catchments and six provenance regions as defined by the Belgian and French authorities.

The geographical distribution of the two cpDNA haplotypes F and G was consistent with the distribution of these haplotypes in the local populations. At the nuclear SSR level, the collection displayed a range of alleles that were, except for a few rare alleles, representative of the different populations, and no significant differentiation between the collection and local populations was observed. No significant difference in genetic diversity (Â, He) was observed according to either the river catchment or the provenance region. As far as the allelic diversity is concerned, therefore, the collection seems suitable for providing trees for river replanting programs, whatever the river catchment or provenance region. However, the lack of differentiation using neutral markers does not preclude the possibility that the populations are locally adapted to their site conditions. Adaptive variation in *A. glutinosa* populations has been found for phenology and growth rate (DeWald and Steiner 1986), and phenotypic variation has been observed (McVean 1953). No adaptive divergence, however, has been found among provenance regions in Flanders in Belgium (De Kort et al. 2014).

Genetic structure in the cross-border area according to geographical criteria

No structure was detected over the sampled area using STRUCTURE software and nuclear SSR data, suggesting that the sampling sites belonged to a same admixed population without any clear genetic structure. The AMOVA conducted on nuclear SSR data with different parameters (population, river catchment, provenance region) indicated a very low level of differentiation. The population parameter, however, displayed the highest level of differentiation.

In contrast, highly significant differentiation among populations, river catchments, and provenance regions was found with the cpDNA markers. Again, the differentiation among populations was the most significant, explaining 75 % of the molecular variance. This result suggests little seed movement between populations.

The various approaches used in this study consistently indicated the lack of a significant geographical structure when using SSR markers at the level of the cross-border area, whereas the distribution of the cpDNA haplotypes appeared to be significantly structured. Particularly striking was the lack of significant differentiation between the two cpDNA haplotypes when analyzed on a nuclear SSR marker basis, despite their strong geographical distribution in the area under study. It is well known that the mode of inheritance of these markers has a major effect on the partitioning of genetic diversity, with maternally inherited markers based on cpDNA (which implies seed dispersal) leading to considerably higher population genetic differentiation than

nuclear biparentally inherited markers (involved in both pollen and seed dispersal) (Petit et al. 2005). In species such as *A. glutinosa*, where the pollen/seed flow ratio is high, a higher migration rate of nuclear markers relative to cpDNA markers is therefore expected.

Although *A. glutinosa* seeds are dispersed mainly by water and the species tends to grow in linear stands along streams and rivers, no particular structure could be related to the river catchments. This seems consistent with the high rate of pollen/seed flow. Therefore, managing the diversity of the species for restoration purposes on the basis of river catchments does not seem appropriate.

Similarly, the lack of genetic differentiation according to provenance regions as currently defined does not seem to justify this criterion for restoration purposes. The distribution of the cpDNA haplotypes, however, reveals a clear cross-border geographic structuration, which should encourage the definition of new, larger provenance regions, independent of national limits. Ecological studies should be conducted in order to test the existence of local adaptation.

Compliance with ethical standards

References

Aguayo J, Elegbede F, Husson C, Saintonge FX, Marçais B (2014) Modelling climate impact on an emerging disease, *Phytophthora alni* induced alder decline. Glob Chang Biol 20:3209–3221

Bassil N, Botta R, Mehlenbacher S (2005) Microsatellite markers in hazelnut: isolation, characterization, and cross-species amplification. J Am Soc Hortic Sci 130:543–549

Beatty G, Montgomery W, Tosh D, Provan J (2015) Genetic provenance and best practice woodland management: a case study in native alder (*Alnus glutinosa*). Tree Genet Genomes 11:92

Bibalani GH, Bazhrang Z, Mohsenifar H, Joodi L (2008) The side roots pulling effect of alder (*Alnus glutinosa*) on river bank soil strong in North Iran. Int J Bot 4:290–296

Boccacci P, Akkak A, Bassil V, Mehlenbacher S, Botta R (2005) Characterization and evaluation of microsatellite loci in European hazelnut (*Corylus avellana*) and their transferability to other *Corylus* species. Mol Ecol Notes 5:934–937

Botstein D, White RL, Skolnick M, Davis RW (1980) Construction of a genetic linkage map in man using restriction fragment length polymorphism. Am J Hum Genet 32:314–331

Claessens H, Oosterbaan A, Savill P, Rondeux J (2010) A review of the characteristics of black alder (*Alnus glutinosa* [L.] Gaertn.) and their implications for silvicultural practices. Forestry 83:163–175

Cubry P, Gallagher E, O'Connor E, Kelleher C (2015) Phylogeography and population genetics of black alder (*Alnus glutinosa* (L.) Gaertn.) in Ireland: putting it in a European context. Tree Gene Genomes 11:99

De Kort H, Mergeay J, Vander Mijnsbrugge K, Decocq G, Maccherini S, Kehlet Bruun HH, Honnay O, Vandepitte K (2014) An evaluation of seed zone delineation using phenotypic and population genomic data on black alder *Alnus glutinosa*. J Appl Ecol 51:1218–1227

Demesure B, Sodzi N, Petit RJ (1995) A set of universal primers for amplification of polymorphic non-coding regions of mitochondrial and chloroplast DNA in plants. Mol Ecol 4:129–131

DeWald L, Steiner K (1986) Phenology, height increment, and cold tolerance of *Alnus glutinosa* populations in a common environment. Silvae Genet 35:205–211

Druart P, Leclercq M, Joussemet MA, Bender J, Adant S, De Le Court B, Mertens P, Hussson C, Chandelier A, Mingeot D, Hausman JF, Evlard A, Bajji M, Delcarte J, Lambot F (2013) Construction et développement d'une filière de production d'écotypes ligneux pour l'application des techniques végétales à la renaturation des berges de cours d'eau. In: Druart P, Husson C, Paul R (eds) Renaturation des berges de cours d'eau et phytoremédiation. Presses agronomiques de Gembloux, Gembloux, pp 121–155

Earl D, vonHoldt B (2012) STRUCTURE HARVESTER: a website and program for visualizing STRUCTURE output and implementing the Evanno method. Conserv Genet Resour 4:359–361

Elegbede CF, Pierrat JC, Aguayo J, Husson C, Halkett F, Marçais B (2010) A statistical model to detect asymptomatic infectious individual with an application in the *Phytophthora alni* induced alder decline. Phytopathology 11:1262–1269

Ennos RA (1994) Estimating the relative rates of pollen and seed migration among plant populations. Heredity 72:250–259

Evanno G, Regnaut S, Goudet J (2005) Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. Mol Ecol 14:2611–2620

Goudet J (1995) FSTAT (vers. 1.2): a computer program to calculate F-statistics. J Hered 86:485–486

Havrdová A, Douda J, Krak K, Vít P, Hadincová V, Zákravsky P, Mandák B (2015) Higher genetic diversity in recolonized areas than in refugia of *Alnus glutinosa* triggered by continent-wide lineage admixture. Mol Ecol 24:4759–4777

Husson C, Aguayo J, Revellin C, Frey P, Ioos R, Marçais B (2015) Evidence for homoploid speciation in *Phytophthora alni* supports taxonomic reclassification in this species complex. Fungal Genet Biol 77:212–214

Jakobsson M, Rosenberg N (2007) CLUMPP: a cluster matching and permutation program for dealing with label switching and multimodality in analysis of population structure. Bioinformatics 23:1801–1806

Kajba D, Gracan J (2003) EUFORGEN technical guidelines for genetic conservation and use for black alder (*Alnus glutinosa*). International Plant Genetic Resources Institute, Rome, 4 pages

King RA, Ferris C (1998) Chloroplast DNA phylogeography of *Alnus glutinosa* (L.) Gaertn. Mol Ecol 7:1151–1161

Kulju K, Pekkinen M, Varviot S (2004) Twenty-three microsatellite primer pairs for *Betula pendula* (Betulaceae). Mol Ecol Notes 4:471–473

Lepais O, Bacles C (2011) De novo discovery and multiplexed amplification of microsatellite markers for black alder (*Alnus glutinosa*) and related species using SSR-enriched shotgun pyrosequencing. J Hered 102:627–632

Lepais O, Muller S, Ben Saad-Limam S, Benslama M, Rhazi L, Belouahem-Abed D, Daoud-Bouattour A, Gammar A, Ghrabi-Gammar Z, Bacles C (2013) High genetic diversity and distinctiveness of rear-edge climate relicts maintained by ancient tetraploidisation for *Alnus glutinosa*. PLoS One 8:e75029

Lowe AJ, Boshier D, Ward M, Bacles C, Navarro C (2005) Genetic resource impacts of habitat loss and degradation; reconciling empirical evidence and predicted theory for neotropical trees. Heredity 95: 255–273

McVean DN (1953) *Alnus glutinosa* (L.) Gaertn. J Ecol 41:447–466

Mingeot D, Baleux R, Watillon B (2010) Characterization of microsatellite markers for black alder (*Alnus glutinosa* [L.] Gaertn). Conserv Genet Resour 2:269–271

Nagy S, Poczai P, Cernák I, Gorji AM, Hegedűs G, Taller J (2012) PICcalc: an online program to calculate polymorphic information content for molecular genetic studies. Biochem Genet 50:670–672

Nybom H (2004) Comparison of different nuclear DNA markers for estimating intraspecific genetic diversity in plants. Mol Ecol 13:1143–1155

Oosterhout CV, Hutchinson W, Wills D (2004) Micro-checker: software for identifying and correcting genotyping error in microsatellite data. Mol Ecol Notes 4:535–538

Peakall R, Smouse P (2006) GENALEX 6: genetic analysis in excel. Population genetic software for teaching and research. Mol Ecol Notes 6:288–295

Persson H, Widén B, Andersson S, Svensson L (2004) Allozyme diversity and genetic structure of marginal and central populations of *Corylus avellana* L. (Betulaceae) in Europe. Plant Syst Evol 244:157–179

Peterjohn W, Correll D (1984) Nutrient dynamics in an agricultural watershed: observations on the role of a riparian forest. Ecology 65: 1466–1475

Petit RJ, Duminil J, Fineschi S, Hampe A, Salvini D, Vendramin GG (2005) Comparative organization of chloroplast, mitochondrial and nuclear diversity in plant populations. Mol Ecol 14:689–701

Prat D, Leger C, Bojovic S (1992) Genetic diversity among *Alnus glutinosa* (L.) Gaertn. populations. Acta Oecol 13:469–477

Pritchard JK, Stephens M, Donnelly P (2000) Inference of population structure using multilocus genotype data. Genetics 155: 945–959

Direction des Ressources Forestières (2011) Dictionnaire des provenances recommandables des essences forestières en Région wallonne CD 55.47 Service public de Wallonie, DGARNE, Département de la Nature et des Forêts, Namur, 41pp. http://environnement.wallonie.be/orvert/docs/Dictionnaire_prov_reco.pdf. Accessed 12 March 2015

Rousselet C, Bilger I, Héois B, Ginisty C (2003) Conseils d'utilisation des matériels forestiers de reproduction: régions de provenance, variétés améliorées. Ministère de l'agriculture de l'alimentation de la pêche et des affaires rurales – DGFAR, Paris

Rousset F (2008) Genepop'007: a complete reimplementation of the Genepop software for Windows and Linux. Mol Ecol Resour 8: 103–106

Schneider S, Roessli D, Excoffier L (2000) Arlequin, version 2.0: a software for population genetic data analysis. User manual ver 2.000. Genetics and Biometry Lab, Dept. of anthropology, University of Geneva, Geneva

Steiner W, Gregorius HR (1999) Incompatibility and pollen competition in *Alnus glutinosa*: evidence from pollination experiments. Genetica 105:259–271

Thoirain B, Husson C, Marçais C (2007) Risk factors for the *Phytophthora*-induced decline of alder in Northeastern France. Phytopathology 97:99–105

Zhuk A, Veinberga I, Daugavietis M, Rungis D (2008) Cross-species amplification of *Betula pendula* Roth. simple sequence repeat markers in *Alnus* species. Balt For 14:116–121