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Genetic differentiation within and among populations of chestnut (*Castanea sativa* Mill.) and wild cherry (*Prunus avium* L.)

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Isoenzymes were used to examine genetic variability within and among populations of chestnut (*Castanea sativa* Mill.) and wild cherry (*Prunus avium* L.). Isoenzymes were sufficiently robust to allow the attribution of chestnut shoots to stumps; other markers were required to confirm the estimations of wild cherry clone sizes. No genetic structure was observed within populations of the two species and no significant departures from Hardy–Weinberg equilibrium were detected for the isoenzymes tested excluding one locus. *F*-statistics and measurements of genetic distances revealed little genetic differentiation among populations of these species. This could be explained by the influence of human activities and the limited number of generations observed for these species since the last glaciation.

Keywords: asexual and sexual propagation, *Castanea sativa* Mill., genetic differentiation, isoenzymes, *Prunus avium* L.

Introduction

The genetic differentiation of plant populations using enzymatic markers, which are assumed to represent neutral genes, has been extensively studied during the last 20 years. Hamrick (1989) showed that plants exhibit a generally high level of intrapopulation diversity for enzymatic markers and an interpopulation diversity related to the life-history traits of the species. For example, tree species have a lower interpopulation differentiation than other plants.

However, it is not known whether this low differentiation is also observed for tree species which do not exhibit special adaptation for seed dispersal such as chestnut (*Castanea sativa* Mill.) and wild cherry (*Prunus avium* L.) These two species are diploid and monoecious. Pollination is performed by insects for both species and by action of wind for chestnut. Fruits are dispersed by humans and animals. Some vegetative propagation of chestnut may be the result of human activity while suckering occurs naturally for wild cherry. Incompatibility systems can prevent self-pollination in both species (Crane, 1923; Clapper, 1954).

In the present study, isoenzymatic genetic markers are used to assess the genetic variability of selected populations of chestnut and wild cherry. Our objectives are: (i) to determine the extent of vegetative reproduction and (ii) to estimate the genetic differentiation within and among populations.

Materials and methods

Sampled populations

Trees were sampled in 1988 from five pure chestnut stands over a wide geographic area in France (Table 1). To determine the extent of vegetative propagation and within population differentiation, 117 trees were sampled in population C1.

Between 14 and 81 trees were sampled from four wild cherry populations in 1988 and 1989 (Table 1). Population W1 was an almost pure stand and was systematically sampled using a 30 × 30 m grid. The mixed stands of populations W2 and W4 were sampled from four and three clumps, respectively. Trees in population W3 were sampled approximately 50 m apart from a clump along the edge of the forest.

Growing shoot tips were collected from population W4. Buds were collected from all other chestnut and

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Table 1 Details of the stands investigated. C: chestnut; W: wild cherry. Lat.: latitude; Long.: longitude; S. treat.: stand treatment; s.c.s: simple coppice system; t.f: timber forest; *N*: number of sampled trees

Stands	Location	Lat.	Long.	S. treat.	<i>N</i>
C1	Angervilliers	48°35'N	2°30'E	s.c.s.	117
C2	Saint Caprais	44°07'N	1°14'E	s.c.s.	15
C3	Saint Mariens	45°08'N	0°24'E	t.f.	38
C4	Alzon	43°58'N	3°27'E	s.c.s.	36
C5	Avants Monts	43°29'N	2°45'E	t.f.	42
W1	St Andre de l'Eure	48°55'N	1°17'E	t.f.	61
W2	Signy l'Abbaye	49°43'N	4°25'E	t.f.	19
W3	Gomaringen	48°26'N	9°08'E	t.f.	14
W4	Sezanne	48°41'N	3°43'E	t.f.	81

wild cherry stands, except from population W2 where buds were collected from ramets in a nursery.

Electrophoretic analysis

Enzyme extractions for chestnut samples were performed according to Frascaria & Lefranc (1992). Gel buffers were described by Meinzel & Market (1967) and staining techniques by Harris & Hopkinson (1976) and Tanksley & Rick (1980). Three isoenzyme loci were studied, including PGI-1 (E.C.5.3.1.9), IDH-2 (E.C.1.1.1.42) and SDH-1 (E.C.1.1.1.25). The Mendelian inheritance of allozymes variants for these loci was determined by Bonnefoi (1984), Villani *et al.* (1986) and Fineschi *et al.* (1990).

Techniques for electrophoresis, the description of isoenzyme variants and the genetic control analysis of wild cherry samples were given in Santi & Lemoine (1990). Ten polymorphic loci were studied: SDH-1, IDH-1, LAP-1 (E.C.3.4.11.1), MDH-1 and MDH-2 (E.C.1.1.1.37), ACP-1 and ACP-2 (E.C.3.1.3.2), GOT-1 and GOT-2 (E.C.2.6.1.1) and TO (E.C.1.15.1.1). GOT-1 and LAP-1 loci are closely linked (Santi & Lemoine, 1991). Three loci, TO (E.C.1.15.1.1), ACP-2, and MDH-2, were only used to aid in the final identification of clones, as their genetic control has not yet been studied successfully.

Statistical analysis

Isoenzymatic markers were used to detect the possible occurrence of vegetative propagation in population C1 and all wild cherry populations. Further discrimination of wild cherry populations was achieved using isoenzyme phenotypes of ACP-2, MDH-2 and TO loci, as well as morphological traits (branching habit and grain of the wood, only for population W2).

For population W1, the probability (*P*) of observing a group of *n* or more trees of a given multiple-loci genotype \underline{A} without vegetative propagation was computed. Assuming that the parents of the sampled trees were part of a population under Hardy-Weinberg equilibrium and with the same allelic frequencies as the sampled population (total sample size = *N*), the probability of the occurrence of one genotype \underline{A}_i (= *aa*, *ab* or *bb* for example) at locus *i* (SDH-1, IDH-1, LAP-1, MDH-1, ACP-1 and GOT-1) was estimated by the frequency of this genotype in our observed sample. Assuming no linkage disequilibrium, the probability of occurrence of a determined combination *A* of six genotypes \underline{A}_i is:

$$P_{\underline{A}} = \pi_{i=1}^6 P_{\underline{A}_i}$$

$P_{\underline{A}_i}$ being the probability of the occurrence of genotype \underline{A}_i for the locus *i* (with *i* as a member of).

In our sample of size 61, the probability of the occurrence of *k* individuals with the same genotype \underline{A} is a binomial probability (assuming no distortion in gametic distribution):

$$P = C_{61}^k (P_{\underline{A}})^k (1 - P_{\underline{A}})^{61-k},$$

and the probability that a group of size *n* or greater occurs with the same genotype \underline{A} is:

$$P = \sum_{k=n}^{61} C_n^k (P_{\underline{A}})^k (1 - P_{\underline{A}})^{61-k}.$$

To determine within-population differentiation, populations C1 and W1 were geographically subdivided into sections of equal areas (called subpopulations). Population C1 was subdivided automatically four ways with a specific computer program (Gouyon, personal communication) into 20 (average size of 5.85 individuals), 12 (9.75 individuals), 11 (10.7 individuals) and 6 (19.5 individuals) subpopulations. Population W1 was subdivided four ways, as the distribution of trees was less homogeneous (without individuals 24 and 17 which were too far away to enter in a group without extending its area), into 7 (average size of 6.9 individuals), 5 (9.6 individuals), 3 (16 individuals) and 2 (24 individuals) subpopulations. Gene frequencies and genotypic data were analysed using the Chi-square test and *F*-statistics (Wright, 1965; Nei, 1977, 1986).

The *Gst* index, identical to the *Fst* index when a single locus is considered (Nei, 1973), was used to evaluate genetic differentiation among populations (Nei, 1978). The genetic distance (Nei, 1972) was calculated between pairs of populations.

Results

Two alleles and three genotypes for each of the three loci studied were recorded in chestnut populations. In

wild cherry populations, two alleles and three genotypes were observed for the IDH-1, LAP-1, MDH-1 and SDH-1 loci, and three alleles and four genotypes for the ACP-1 and GOT-1 loci. Only one tree (22 in population W1) contained a single allele of GOT-2. Two phenotypes were recorded for MDH-2 and TO loci and eight for ACP-2.

Detection of vegetative propagation

Old chestnut stumps were sometimes so large it was not clear whether they were composed of more than one genotype. Doubtful cases were therefore tested using the three polymorphic loci. By this method, shoots were attributed to the correct stump.

In wild cherry populations, when we examined the distribution of trees within a given group (Figs 1 and 2), the same genotype was found to occur in clusters, presumably representing ramets arising from a single clone through suckering. Furthermore, all trees of a given group were heterozygotes for at least one locus (except for group 96-97 in W4), which excludes local monomorphism as an explanation.

In population W1, 8 groups with identical genotypes were found among 61 trees. Most of them were geographically gathered, which suggested a clonal origin (groups 40-41, 46-47 and 49-50; Fig. 1), but remembering that trees were sampled 30 m apart, the probability of obtaining through sexual reproduction a homogeneous group of at least size n (n = observed



Fig. 1 Spatial distribution of trees in wild cherry populations W1 and W2. Numbers refer to individual trees, represented as dots, heavy lines indicate the position and the limits of the supposed clone groups and dotted lines indicate trees which are too widely separated to be included in the supposed clone groups: tree no. 9 from the clone group no. '5-6-7-8-10', tree no. 20 from tree no. 21, tree no. 24 from tree no. 25, and tree no. 45 from the clone group no. '46-47'.

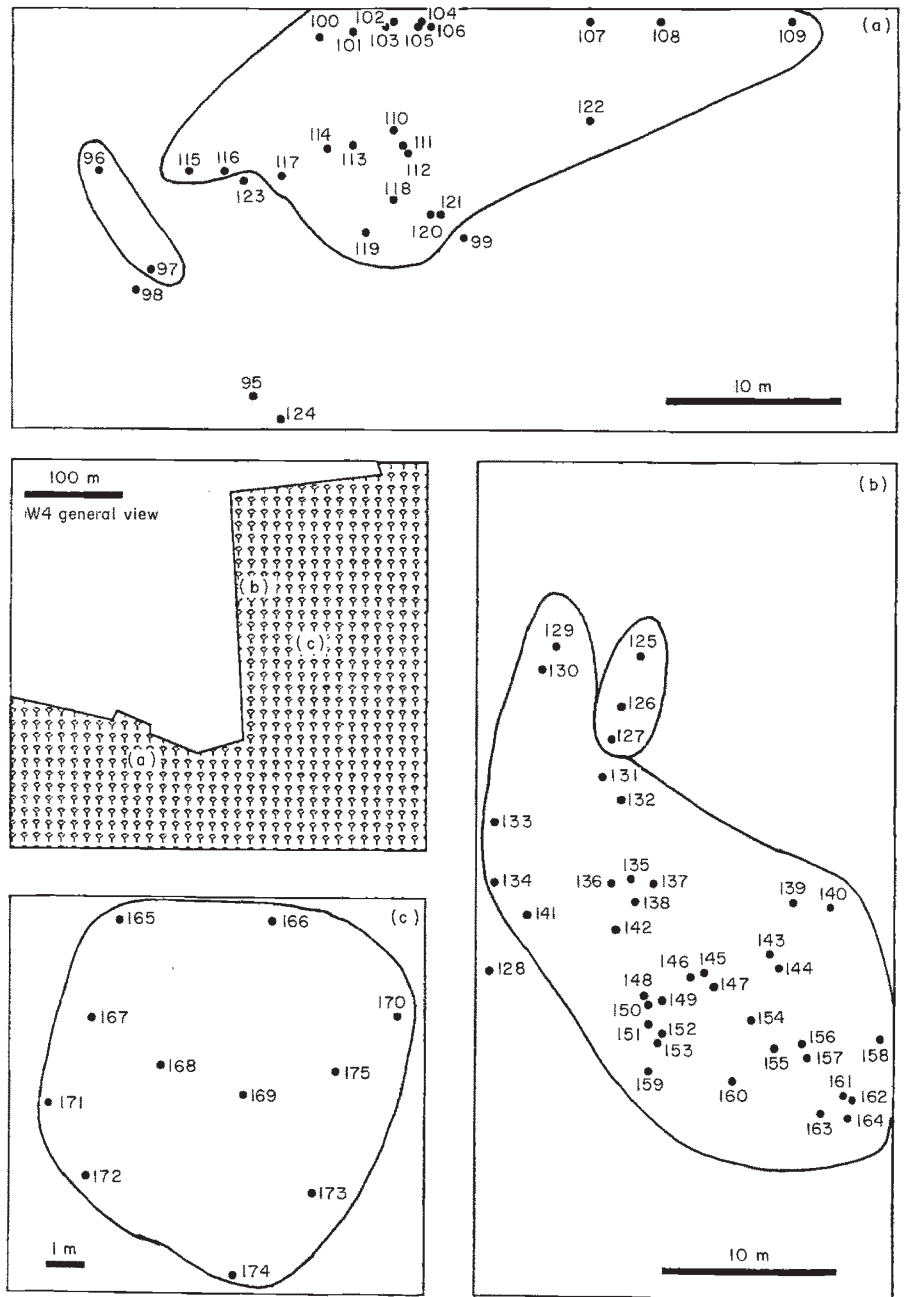


Fig. 2 Spatial distribution of the trees in wild cherry population W4. Numbers refer to individual trees, represented as dots, heavy lines indicate the position and the limits of the supposed clone groups.

size) was estimated for other groups (groups 5–10, 13–14, 20–21, 24–25, 45–47 and 59–61). This probability was small for groups including trees 5–10 ($P = 0.004$, excluding tree no. 9), 13–14 ($P = 0.06$) and 59–60 ($P = 0.0001$). For other groups tested, higher probabilities were obtained ($P > 0.1$). In these cases, the occurrence of vegetative propagation was uncertain. Also, trees no. 9, 20 or 21, 24 or 25 and 45 were too far away from their identical clone group (90–160 m) to be included (Fig. 1). Therefore, 50 rather than 61 individuals were used for the within population differentiation analysis.

In population W2, 7 trees from group A (73–79) had the same multiple-locus genotype, while all trees from the other three groups had different genotypes (Fig. 1). Group A may represent a single clone, but morphological features (data not shown) indicated that this group probably included two subgroups (groups 73–74 and 75–79).

All 24 trees sampled in population W3 had different genotypes.

In population W4, 3 groups of trees were sampled within the stand (Fig. 2). Among these groups, the individuals sampled appeared to represent only a

few clones. In group A, only 7 genotypes were found among 30 trees (23 adjacent trees had the same genotype). In group B, only 3 genotypes were found among 30 trees (one genotype was represented by 26 trees). In group C, all 21 trees had the same genotype.

Table 2 indicated the estimated size of clone groups identified in the 3 populations studied. It is possible that more genotypes may be detected within the same clonal group by testing additional genetic markers.

Table 2 Rough estimations of wild cherry clone size in populations W1, W2 and W4

Populations	Clone groups	Number of trees	Clone sizes (m ²)
W1	5-10	6	5200
	13-14	2	1700
	20-21	2	2400
	40-41	2	1000
	46-47	2	1400
	49-51	2	1700
	59-61	2	1700
W2	73-74	2	300
	75-79	5	1000
W4	96-97	2	25
	100-122	23	600
	125-127	3	20
	129-164	36	600
	165-175	11	100

However, as stated previously, we assumed that all trees belonging to the same group represent one clone produced by suckering. Hence, selected data were chosen for subsequent analysis of variability; only a single tree, from each supposed clone in population W1, W2 and W4, was used for the estimation of allelic frequencies.

Within population differentiation

Little differentiation was observed between subpopulations (mean *Fst* < 7.9 per cent for C1 and < 9.2 per cent for W1) (Table 3). In neither population was a genetic structure due to sexual reproduction detected; the neighbourhood size, as defined by Wright (1951), was probably larger than the chosen population size.

There was no evidence for excess heterozygosity in all loci, except for the SDH-1 locus in population C1 (χ^2 significant at the 0.1 level, Table 3). *Fis* values were generally low among the largest subpopulations. These results suggested an influence of sample size, depending on the groupings.

Genetic differentiation among populations

Although significant differences in allele frequencies among populations C1 to C5 and W1 to W4 were detected for three loci (Table 4), estimated values of *Gst* and *Fst* were low (*Fst* mean = 2.7 per cent for chestnut populations and 4.9 per cent for wild cherry

Table 3 Genetic structure of chestnut C1 and wild cherry W1 populations

Partition number	Chestnut				Wild cherry							
	PGI-1	IDH-2	SDH-1	mean	SDH-1	IDH-1	LAP-1	MDH-1	ACP-1	GOT-1	mean	
Genic structure												
χ^2 test	1	NC	NC	NC	—	NC	NC	NC	NC	NC	NC	—
	2	NC	NC	NC	—	NC	NC	NC	NC	NC	NC	—
	3	0.567	0.089	9.377*	—	3.30	4.20	2.90	3.70	NC	NC	—
	4	0.555	0.079	9.870*	—	3.60	2.80	1.10	2.00	2.0	0.10	—
Genotypic structure												
<i>F</i> -statistics												
<i>Fst</i>	1	0.083	0.113	0.043	0.079	0.114	0.049	0.056	0.059	0.153	0.040	0.080
	2	0.070	0.065	0.063	0.066	0.272	0.045	0.032	0.031	0.122	0.050	0.092
	3	0.040	0.047	0.036	0.041	0.031	0.040	0.027	0.034	0.015	0.018	0.027
	4	0.023	0.026	0.020	0.023	0.034	0.027	0.011	0.019	0.019	0.001	0.018
<i>Fis</i>	1	-0.165	-0.142	-0.326	-0.211	-0.047	-0.076	0.077	-0.255	-0.352	-0.080	-0.122
	2	-0.135	-0.055	-0.332	-0.174	0.031	-0.073	0.088	-0.201	-0.298	-0.075	-0.088
	3	-0.119	-0.054	-0.320	-0.164	0.026	-0.068	0.092	-0.206	-0.158	-0.039	-0.058
	4	-0.091	-0.029	-0.300	-0.140	0.023	-0.053	0.107	-0.187	-0.162	-0.021	-0.048

*Significant at the 0.1 level, NC = non-calculable.

Table 4 Genetic structure of chestnut (C1–5) and wild cherry (W1–4) populations (pop)

Chestnut					Wild cherry								
Pop		PGI-1	IDH-2	SDH-1	mean	Pop	SDH-1	IDH-1	LAP-1	MDH-1	ACP-1	GOT-1	mean
Genic structure													
Allele frequencies													
C1	a	0.423	0.829	0.301	—	W1	0.227	0.527	0.609	0.282	0.874	0.836	—
	b	0.577	0.171	0.699	—		0.773	0.473	0.391	0.718	0.126	0.156	—
	c	—	—	—	—		0	0	0	0	0	0.008	—
C2	a	0.733	0.730	0.125	—	W2	0.321	0.679	0.536	0.250	0.750	0.857	—
	b	0.267	0.027	0.875	—		0.679	0.321	0.464	0.075	0.179	0.143	—
	c	—	—	—	—		0	0	0	0	0.071	0	—
C3	a	0.526	0.636	0.217	—	W3	0.429	0.607	0.786	0.214	0.750	0.964	—
	b	0.474	0.364	0.783	—		0.571	0.393	0.214	0.786	0.025	0.036	—
C4	a	0.458	0.819	0.454	—	W4	0.577	0.923	0.962	—	—	1.000	—
	b	0.542	0.181	0.546	—		0.423	0.077	0.038	—	—	0	—
C5	a	0.488	0.819	0.296	—	—	—	—	—	—	—	—	—
	b	0.512	0.181	0.704	—	—	—	—	—	—	—	—	—
χ^2 test		23.75*	24.14*	23.31*	—		13.8*	14.5*	15.9*	0.56	NC	NC	—
Genotypic structure													
<i>F</i> -statistics and <i>Gst</i>													
<i>Fst</i>		0.023	0.035	0.022	0.027		0.072	0.075	0.083	0.003	0.022	0.037	0.049
<i>Gst</i>		0.034	0.026	0.140	0.066		0.071	0.101	0.136	0.004	0.020	0.059	0.065
<i>Fit</i>		0.086	0.024	-0.260	-0.050		0.063	0.033	0.220	0.175	-0.102	0.067	0.018
<i>Fis</i>		0.064	-0.010	-0.289	-0.078		-0.010	-0.046	0.150	-0.179	-0.127	0.031	-0.030

*Significant at the 0.1 level, NC = non-calculable.

Table 5 (a) Calculations of Nei's genetic distances among five chestnut populations (Nei, 1972). (b) Calculations of Nei's genetic distances among four wild cherry populations (Nei, 1972)

(a) Populations	C2	C3	C4	C5
C1	0.072	0.027	0.017	0.002
C2		0.035	0.107	0.053
C3			0.067	0.022
C4				0.015
(b)	W2	W3	W4	
W1	0.016	0.034	0.155	
W2		0.035	0.114	
W3			0.049	

populations, Table 4), indicating a limited level of differentiation among the populations.

Nei's genetic distances calculations (Table 5) also suggested that there was little genetic differentiation among the populations. A close genetic relationship existed among C1, C4 and C5, while W4 was slightly more separated from other wild cherry populations

(Table 5), though geographic and genetic distances were never correlated.

Discussion and conclusion

The isoenzyme loci used to assess chestnut populations appear to be sufficient to allow the attribution of shoots of stumps. In wild cherry populations, the genetically homogeneous groups observed in our study cover a maximum of 0.52 ha but other genetic markers are certainly necessary to estimate precisely the size of the sucker groups. This estimated value is far less than that observed with *Populus tremuloides* (Cheliak & Dancik, 1982), but is in agreement with observations made on *Prunus avium* populations which occur in small dispersed clusters. *Prunus avium* has less suckering ability than *Prunus cerasus*, a closely related species with no incompatibility system (Crane, 1923). In natural populations of *Prunus avium*, individuals with a high suckering ability can produce more gametes, but have a higher probability of producing somatic mutations for the gametophytic incompatibility S-locus. Spontaneous and artificially obtained mutations on the S-locus (restoring in this case self-compatibility) have been observed by Lewis & Crowe (1954). A mutant for

another allele of the S-series can easily pollinate all of the clusters producing highly homozygous progeny with a supposed depressed fitness. The existence of the incompatibility system could therefore explain the small size of the suckering groups in the populations studied.

The *Fis* fixation indices calculated for the two species studied suggest that no genetic structure exists within the populations sampled. Negative *Fis* values found for one locus might be the result of either the selection of closely linked loci or the differing male and female contributions towards seed production, which could favour the survival of heterozygotes.

Lack of differentiation among chestnut populations agrees with the results of studies conducted in Italy on the same species (*Fst* = 6.5 per cent, Villani *et al.*, 1991) and in other species of Fagaceae (*Fst* = 4.5 per cent for *Quercus ilex*, Yacine & Lumaret, 1988; 4.2 per cent for *Quercus petraea* and *Quercus robur*, Kremer *et al.*, 1991; 5 per cent for *Alnus crispa*, Bousquet *et al.*, 1987; 6 per cent for *Fagus sylvatica*, Cuguen *et al.*, 1989). The lack of differentiation observed among wild cherry populations is homologous to that found among *Ficus carita* populations (*Fst* = 4.3 per cent, Valizadeh, 1978), which have a similar population distribution and dispersal factors.

According to Loveless & Hamrick (1984), species whose seeds are dispersed by gravity (chestnut) or by animals (wild cherry) have a mean *Gst* of 0.446 and 0.332, respectively, far greater than our calculated values. Nevertheless, it is remarkable how little migration is required to prevent genetic divergence among populations, resulting from random genetic drift. The effect can be seen quantitatively by considering the Wright's Island model; the *Fst* at equilibrium is equal to $(1/1 + 4mN)$, provided that *m* remains low (*N* = population size, *m* = migration rate, Wright, 1951). This equation implies that the *Fst* decreases as the number of migrants and the population size increase. Indeed, the decrease of *Fst* with increasing *Nm* is extremely rapid. The population size *N* is high for long-lived, late successional species, such as chestnut and wild cherry. These two species have low pollen production (almost for wild cherry, Pouvreau, personal communication) and, at first glance, limited seed dispersal. The expected low migration rate should therefore lead to a higher level of population variability than in tree species whose pollen and/or seeds are wind dispersed (Loveless & Hamrick, 1984). However, the migration rate may be higher than expected since human activities may have greatly influenced the migration of seeds produced by edible species (Pigliucci *et al.*, 1990; Frascaria & Lefranc, 1992) and probably because wild cherry pollen is easily located by insects and able to

travel 1 or 2 km. The migration rate *m* is therefore not low enough to compensate for the effect of a large population size. In addition, the number of generations completed by European trees is limited, since the species expanded out of refugia following the last glaciation (10,000 years ago) and the *Fst* has not yet reached equilibrium (Hiebert & Hamrick, 1982).

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