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SSR-based analysis of clonality, spatial genetic structure and introgression from the Lombardy poplar into a natural population of *Populus nigra* L. along the Loire River

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Abstract:

A scarcity of favourable habitats and introgression from exotic cultivars are two major threats to black poplars (*Populus nigra* L.) in Europe. Natural vegetative propagation contributes to maintenance of the species in areas where seedling recruitment is limited. Exhaustive sampling of all mature trees in a natural *P. nigra* stand (413 individuals at recorded positions), genotyping at 11 SSR loci, and a standardized analysis framework resulted in a precise description of clonality in terms of (a) frequency, (b) spatial growth form, and (c) impacts on the overall spatial genetic structure (SGS). The high proportion of replicated genotypes detected resulted in a genotypic richness (*R*) of 0.47. Up to 18 ramets were found per multilocus lineage (MLL), but 95% of MLLs contained fewer than five ramets (Pareto index $\beta = 1.07$). No significant difference in vegetative propagation potential was found between genders. Uneven spatial distribution of ramets, with clustering of clonal ramets (aggregation index $A_c = 0.62$) and near-zero intermingling between MLLs (clonal dominance index $D_c = 0.99$), resulted in a 'phalanx' clonal growth form, explaining most of the SGS observed over short distances (0–20 m, $Sp = 0.0324$). Although they did not exhibit the typical columnar shape of the Lombardy poplar (*P. nigra* var. *italica*), five trees were found to be probable F_1 hybrids of this old and widely distributed cultivar.

Keywords : *Populus nigra* ; Lombardy poplar ; Clonality ; Spatial genetic structure ; Introgression ; Clonal growth form

Introduction

Various subspecies of black poplar (*Populus nigra* L.) have been proposed on the basis of morphological traits; however, variation may be the result of the species' wide distribution, ranging from the British Isles to Western Asia and from the Mediterranean coast of Africa to Northern Europe, excluding Scandinavia (Dickmann and Kuzovkina 2008). This pioneer species is found in the early successional stages of riparian woodlands and is considered an indicator of the health and biodiversity of these ecosystems (Rotach 2004). Although *P. nigra* has little commercial use *per se*, it is considered a key species in numerous European breeding programmes. In 2009, 66% of the poplar cuttings sold by French nurseries were *P.* × *euramericana* Dode interspecific hybrids (Paillassa 2010), resulting from crossing male black poplars with female American eastern cottonwoods (*P. deltoides* Bartr.).

The species is threatened by extinction in several parts of its natural range as a result of agriculture, urbanization and other human activities, which have altered both the area available for colonization and the dynamics of floodplains, thus hindering seed dispersal and germination and favouring latter successional hardwood trees (Lefèvre et al. 1998). Even though *P. nigra* is classified as being of '*Least Concern*' in the IUCN red list of threatened species (IUCN 2010), it is thought that there are, for example, only about 7000 trees left in Great Britain, and of these only about 600 are females (Cooper 2006). Recent surveys in the North-Western part of its range indicate that the species survives mainly as scattered relicts, most of which were vegetatively propagated and planted by humans (Koskela et al. 2004; Smulders et al. 2008b). National programmes for the conservation of genetic resources have been established in many European countries, under the collaborative EUFORGEN (European Forest Genetic Resources)

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programme (Frison et al. 1995), making black poplar a model species for *ex-* and *in-situ* conservation genetics (Lefèvre et al. 2001b).

Like most poplar species, black poplar is dioecious and anemophilous. The seeds are released in considerable numbers, they have virtually no dormancy and need a substrate that is continuously wet for a four-week period to allow them to settle and establish (Guilloy-Froget et al. 2002). *P. nigra* is also capable of vegetative propagation when biotic (*e.g.*, human, birds) or abiotic (*e.g.*, flood, wind) disturbances lead to the stimulation of dormant primordia in the roots and shoots of either damaged plants or translocated fragments (Barsoum 2002). Levels of clonality ranging from 0 to 97% (*i.e.*, proportions of sampled trees with identical genotypes) have been reported in several natural European *P. nigra* populations (Arens et al. 1998; Barsoum 2002; Barsoum et al. 2004; Cottrell et al. 1997; Koskela et al. 2004; Legionnet 1997; Pospiskova and Bartakova 2004; Pospiskova and Salkova 2006; Rathmacher et al. 2010; Smulders et al. 2008b; Storme et al. 2004; Winfield et al. 1998).

To facilitate rigorous studies of population and conservation genetics, the frequency, spatio-temporal dynamics, and impacts of clonality must be known. Failing to consider clonality in studied populations can lead to erroneous conclusions, particularly when only a few genotypes predominate or when the sampling schemes used are inappropriate as a result. In addition, both theoretical and empirical studies have highlighted the ecological significance and evolutionary implications of clonality. Because vegetative regeneration is possible even when seedling establishment is impaired or rare, new habitats can be utilized and recovery from disturbances can commence; this has been extensively documented in the American aspen, *P. tremuloides* (Mock et al. 2008). Although no general trend has been established, it

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has been suggested that clonality affects population genetics parameters such as effective population size, linkage disequilibrium, and heterozygosity (Balloux et al. 2003; Yonezawa et al. 2004). At the local scale, uneven spatial distribution of clonal ramets can generate spatial genetic structure (SGS) in established populations (Reusch et al. 1999). Clonality-driven SGS can have important consequences for reproduction in dioecious or self-incompatible species (Charpentier 2002). SGS can also occur in the absence of clonality as a consequence of limited gene dispersal (Epperson 2007; Vekemans and Hardy 2004) or selection in heterogeneous environments (Epperson 1990).

Cultivated poplars are considered to represent another threat to *P. nigra* in Europe; there are two reasons for this. First, they have the same water and soil requirements as autochthonous *P. nigra* populations, thus leading to habitat exclusion (Lefèvre et al. 2001a). Second, gene flow from cultivated trees may lead to introgression (also known as "introgressive hybridization") from exotic species such as *P. deltoides* or *P. trichocarpa* or from allochthonous *P. nigra* gene pools (Cagelli and Lefèvre 1995; Vanden Broeck et al. 2005). *P. nigra* cv. Italica Du Roi (synonymous with *P. pyramidalis* Rozier, *P. italica* (Du Roi) Moench, and *P. fastigiata* Foug.), also known as the Lombardy poplar, is certainly the most ancient poplar cultivar and the one with the widest distribution. Although it was first reported in Lombardy, Italy, at the very beginning of the XVIIIth century, there has been some speculation about its origins (Wood 1994), the two main options being (i) that a mutation in *P. nigra* occurred in Italy and (ii) that it was introduced to Italy from Central Asia. Its timber has been used for building, but its columnar shape also makes it a notable visual element in the landscape. Five cuttings were introduced to France in 1745 and the first plantings were

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along the Loing canal (~100km from our study site) (Pelée de Saint-Maurice 1762) before Napoleon I promoted its planting across the Empire (Stettler 2009). It was introduced into England in 1758, and into the United States in 1784 (Wood 1994). Nowadays, despite its poor timber quality, the Lombardy poplar is commonly found in rural and urban landscapes across the temperate zone. It is currently unclear whether *P. nigra* cv. Italica is a single clone or if it comprises several genotypes that all exhibit the distinctive columnar habit. Although most Italica-like trees are males, a few female columnar *P. nigra* trees or cultivars have been reported, one of them being *P. nigra* L. cv. Thevestina (Dode) Bean. The reference cultivar found in the International Poplar Commission database (http://www.populus.it/) is a male and is referred to as "San Giorgio". The Lombardy poplar is densely branched and is often planted as windbreaks or as single trees; thus it is supposed to be a major pollen producer. Moreover, since the cultivar is part of the *P. nigra* species, barriers against introgression into autochtonous wild *P. nigra* populations could be assumed to be low. A few previous studies have, however, reported introgression levels (*i.e.*, the proportion of potential F_1 siblings originating from Italica) of only 0 – 1.6% (Imbert and Lefèvre 2003, Tabbener and Cottrell 2003, Vanden Broeck et al. 2004).

Here, we report on an exhaustive sampling strategy involving accurate geopositioning and SSR genotyping in a natural population belonging to a European Intensive Study Site (EVOLTREE ISS Loire – Zone 4) and representative of numerous *P. nigra* populations in France (*i.e.*, mature populations with significant levels of anthropogenic disturbance). Beyond the usual genetic diversity estimates, major outputs from this study include: (i) the quantification and spatial description of clonality using a recently defined standardized analysis framework; (ii) an evaluation of the proportion of

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SGS that is attributable to the clonal growth form; and (iii) the identification of Lombardy poplar introgression events with high confidence levels.

Materials and methods

Study site and plant material

The study site (7 ha, 915 m long) is located alongside the Loire River near the city of Saint-Ay, France (47°51'N / 1°45'E) (Fig. 1). Part of it belongs to the Saint-Mesmin French National Natural Reserve. Aircraft laser altimetry (data from Direction Régionale de l'Environnement de l'Aménagement et du Logement, Service Loire et Bassin Loire Bretagne, Orléans, France, 2002) revealed a curvilinear depression suggestive of a past meander of the river (Electronic Supplementary material 1). We**,** therefore**,** hypothesize that most of the study site originates from a sandy island that once merged with the riverbank. This is a common phenomenon on this dynamic river system (Gautier and Grivel 2006). Aerial pictures from 1949 onwards (public domain data from Institut Géographique National, Paris, France) reveal that: (i) the merging occurred before 1949; (ii) mature *P. nigra* trees, although at lower densities, have been present since 1949; and (iii) the study area has not been cultivated during that period. Anthropogenic disturbance, however, is highly probable in this suburban area. It may have taken several forms, such as grazing, cutting fodder or fuel wood, dumping garden waste, and path clearing. Clearing is particularly obvious in the north-eastern extremity of the study site. The land adjacent to the river floods frequently, but most of the study site (north of the path) is located above usual flood level. Capillarity, however, can lead to temporary water accumulation in the lowest points of the depression during very

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severe flood events (Saint-Mesmin French National Reserve Administrator, pers. comm.). Black poplars represent at least 75% of the trees in the study area (amounting to 60 trees / ha). They are not restricted to this area as mature trees can be found on both sides of the river and also on most of the islands located nearby. Willows (*Salix alba* L.) compete with black poplars on the bank of the river. Other pioneer - and interestingly alien - tree species are found as scattered individuals **(***Juglans regia* L., *Acer negundo* L.) or groups of trees (*Robinia pseudoacacia* L., *Prunus mahaleb* L.). Although considered to be post-pioneer species, the other trees that are present (*Quercus robur* L., *Acer platanoides* L., *Acer pseudoplatanus* L., *Acer campestre* L, *Fraxinus excelsior* L.) are also indicative of an open-habitat. As expected in such an open space with heterogeneous soil conditions, more than 40 herbs, grasses and shrubs have been identified (Saint-Mesmin French National Reserve Administrator, pers. comm.). A significant part of the ground flora is indicative of high nitrogen availability (*Urtica dioica* L., *Lamium maculatum* L., *Galium aparine* L.). Hygrophilous species such as *Iris pseudacorus* L., *Glechoma hederacea* L. and *Agrostis stolonifera* L. are restricted to the flood-prone areas (south of the path), since water availability declines sharply with elevation.

Except for a few seedlings immediately adjacent to the river, juvenile trees were absent. All sexually mature trees were inventoried (Fig. 1) and their location determined by triangulation using a DT610 electronic digital theodolite (Sokkia Topcon, Mâcon, France). When this technique could not be applied because of topographical constraints, a S500 centimeter precision surveying system was used instead (Leica Geosystems, Le Pecq, France). Sex was determined by looking at the flowers at various dates (>1 observation date per individual).

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Height, using a Forestor Vertex dendrometer (Haglöf Sweden AB, Långsele, Sweden), and girth at breast height were recorded for all studied trees. In the case of multi-stemmed trees (*i.e.*, forking below breast height, or clumped trees with several trunks sprouting from a common base), the girth of each stem was measured and the maximum value recorded. Both parameters exhibited relatively Gaussian distributions. Height and girth ranged from 5.2 to 31.7 m and from 25 to 409 cm, respectively, and the two parameters were highly correlated (Electronic Supplementary Material 2).

Tree ages were assessed for a sample of 20 single-stemmed individuals covering most of the observed range of variation in girth (individuals exceeding 250 cm girth could not be evaluated due to technical constraints). Increment core samples were collected at breast height. After drying, transverse longitudinal sections were cut from each core. Because core analysis of black poplar wood is very difficult, two assessors counted tree rings in a double-blind manner using 6x magnifying lenses. Microscopic analysis did not improve reliability since false-rings were even more likely to be mistaken for true rings. The mean divergence between operators was 20%, and the resulting tree ages (averages of the two estimates) varied between 9.5 and 52.5 years (Electronic Supplementary Material 3). The overall correlation with girth was sufficiently strong ($r_{Spearman} = 0.82$, Electronic Supplementary Material 3) to consider girth ranking as a good predictor of age ranking, at least for single-stemmed individuals.

Many Lombardy poplars have been identified on both sides of the Loire River, in the urban area surrounding the study site, and within the study site itself. All of them are clearly planted ornamental trees. Thirteen large individuals close to the study site were selected for genotyping (Fig. 1). Eleven of these were located on a campsite south-west of the study area ($153 \leq$ girth ≤ 255 cm). Core-analysis was conducted on one of

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these (girth = 212 cm), and the resulting age estimate was 34.5 years. The two other individuals studied were growing very close to each other on the northern edge of the study site (girth = 130 and 134 cm).

Young fresh leaf material was collected from the thirteen Lombardy poplars and the 413 *P. nigra* trees in the inventory and stored at -80 °C whilst awaiting DNA extraction. Each stem of clumped trees was sampled to verify that they represented a single genotype.

DNA extraction and SSR analysis

DNA was extracted from single leaves using a DNeasy 96 Plant Extraction kit (Qiagen, Courtaboeuf, France) according to the manufacturer's instructions.

Genotyping was based on the following 11 unlinked nuclear SSRs (with their corresponding linkage group): PMGC2852 (I), PMGC667 (II), PMGC486 (III), PMGC2235 (IV), PMGC2838 (V), PMGC2578 (VI), PMGC61 (VIII), PMGC333 (XI), PMGC14 (XIII), PMGC433 (XVI) (http://poplar2.cfr.washington.edu), and WPMS05 (XII) (Smulders et al. 2001; Van der Schoot et al. 2000).

The Polymerase Chain Reaction was carried out in a volume of 10 µL, which contained 1 µL template DNA and 9 µL of the following mix: 1× PCR buffer, 1.5 mM $MgCl_2$, 62.5 µM dNTPs mix (all from Invitrogen, Cergy-Pontoise, France), 0.2 µM primers (Eurofins MWG Operon, Ebersberg, Germany), 0.02 µM fluorescently labelled forward primer with either 6-FAM, HEX (Eurofins MWG Operon) or NED (Applied Biosystems, Courtaboeuf, France) fluorescent dyes, and 0.25 U *Taq* polymerase (Invitrogen). Amplification was conducted in a GenAmp 9700 thermocycler (Applied

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Biosystems) for 30 cycles**,** each with the following profile: a 30 s DNA denaturation step at 94 °C, a 30 s annealing step at 50 or 55 °C depending on primers, and a 60 s extension step at 72 °C. The final extension step was extended to 6 min.

As the last denaturation step, a mix containing 2 µL amplified DNA, 7 µL Formamide and 0.25 µL 400HD-Rox size marker (Applied Biosystems) was maintained at 95 °C for 3 min. The fragment separation was then performed in an ABI Prism 3100 Genetic Analyser (Applied Biosystems). The software GENOTYPER 3.7 (Applied Biosystems) was used to score the SSR data.

Clonality detection and description

Identification of multilocus genotypes (MLG) and multilocus lineages (MLL) was based on procedures implemented in GENCLONE 2.0 (Arnaud-Haond and Belkhir 2007) and followed the standardized method proposed by Arnaud-Haond et al. (2007).

The genotypic resolution associated with each possible combination of analysed loci was computed as the resulting number of distinct MLGs (Arnaud-Haond et al. 2005).

Keeping only one ramet per identified MLG, and taking into account departures from Hardy-Weinberg equilibrium as measured by Wright's inbreeding coefficient (F_{is}), the probability (p_{gen}) of occurrence of each observed genotype was estimated according to Young et al. (2002)**:**

$$p_{gen}(F_{is}) = \prod_{i=1}^{l} \left[\left(f_i g_i \right) \left(1 + z_i F_{is(i)} \right) \right] 2^h$$

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where l is the number of loci, h is the number of heterozygous loci, f and g are 'round-robin' allelic frequency estimates of the observed alleles f and g at the i^{th} locus, and $z_i = 1$ (or -1) if the i^{th} locus is homozygous (or heterozygous).

When n ramets with a genotype identical to a previously encountered MLG are detected in a sample population (N), the probability (p_{sex}) of these being derived from distinct reproductive events can be estimated following Parks and Werth (1993)**:**

$$p_{sex}(F_{is}) = \sum_{i=n}^{N} \frac{N!}{i!(N-i)!}\left[p_{gen}(F_{is})\right]^{i}\left[1-p_{gen}(F_{is})\right]^{N-i}$$

The significance of p_{sex} was considered from the first re-encounter ($n = 1$).

To ascertain the uniqueness of MLGs with missing data (*i.e.*, unamplified loci), such MLGs were examined on a case-by-case basis after removing the missing loci from the entire dataset. Based on the recalculated p_{sex} estimates, these MLGs were either designated as being unique or were pooled with another MLG into a MLL. Although somatic mutations can be hypothesized, a similar approach was used to group MLGs that differed at only one locus into MLLs, in order to account for possible scoring errors.

The genotypic richness (R) of the population was computed as $R = (G - 1) / (N - 1)$ where G is the number of MLLs, and N the number of sampled trees (Dorken and Eckert 2001).

For subsequent analyses at the MLL level, $MLL_{\geq 3}$ (with three or more ramets) were reduced to their dominant genotype while $MLL_{=2}$ (with two ramets) were assigned either (i) the heterozygous genotype at the mismatching locus if the other genotype was homozygous (*i.e.*, accepting the miscoded homozygote hypothesis) or (ii) the genotype with the most frequent allele at the locus that differed (*i.e.*, accepting the somaclonal mutation hypothesis).

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In order to characterize the MLL size (N_R, number of ramets) frequency distribution, a cumulative function of the Pareto distribution was fitted to the data as proposed by Arnaud-Haond et al. (2007). This function takes the form $F_{\geq X} = const \,.\, X^{-\beta}$ where $F_{\geq X}$ is the frequency of ramets belonging to a $MLL_{\geq X}$ (with X or more ramets). The shape parameter (β), also called the patchiness exponent, measures the relative importance of large *vs.* small MLLs. β increases exponentially with increasing evenness of distribution. A graphical representation of $log\,(F_{\geq X})$ *vs.* $log\,(X)$ and its associated coefficient of determination r^2 were generated to check the quality of the Pareto approximation.

Two spatial descriptors were computed for each MLL: (i) d_{max}, the maximum distance between ramets, and (ii) $\bar{d}_{neighb.}$, the average distance between nearest neighbours. The relationships between N_R and these two parameters were investigated.

The aggregation index (A_c) proposed by Arnaud-Haond et al. (2007) was calculated using GENCLONE 2.0 as:

$$A_c = (p_{sg} - p_{sp}) / p_{sg}$$

where p_{sg} is the average probability of clonal identity of all sample unit pairs and p_{sp} is the average probability of clonal identity among pairwise nearest neighbours. The significance of A_c was assessed by a 10000-permutation test.

In order to quantify the degree of intermingling between MLLs, the clonal dominance index (D_c) was calculated following Ohsako (2010) for each $MLL_{\geq 3}$ as:

$$D_c = (N_R - 1) / (N_T - 1)$$

where N_R is the MLL size (number of ramets) and N_T is the total number of trees present within the minimal convex envelope containing all ramets of the MLL.

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Detection of introgression from the Lombardy poplar

CERVUS 3.0.3 (Marshall et al. 1998) was used to detect potential F_1 hybrids of the Lombardy poplar in the identified MLLs. The multilocus profile of each Lombardy poplar tree examined was tested for parentage assignment by simple exclusion (Jones and Ardren 2003). The following two criteria were applied for each pairwise comparison: (i) a minimum of eight typed loci in common, and (ii) a maximum of one mismatch corresponding to putative false homozygote coding. Individual probabilities of non-exclusion ($p_{non\text{-}excl.}$) with both parents unknown were calculated using CERVUS 3.0.3 according to Jamieson and Taylor (1997).

Genetic diversity

Deviations from a 1 : 1 sex-ratio were assessed at tree and MLL levels using chi-square tests.

ARLEQUIN 3.5 (Excoffier et al. 2005) was used to compute neutral genetic diversity parameters at the MLL level: observed (H_o) and expected (H_e) heterozygosities (Nei 1978), number of alleles per locus (A), effective number of alleles per locus (A_e) (Hartl and Clark 1997), and Wright's inbreeding coefficient (F_{is}) per locus and sample (Weir and Cockerham 1984). Departures from Hardy-Weinberg equilibrium were revealed by bilateral exact tests on F_{is}.

Spatial genetic structure

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SGS was explored by spatial autocorrelation analysis using GENCLONE 2.0. Multilocus kinship coefficients (F_{ij}) according to Loiselle et al. (1995) were computed for all pairs of sampling units (*i.e.*, trees or MLLs). F_{ij} values were averaged within a given distance class d to produce $\overline{F}_{(d)}$ values. In an isotropic bi-dimensional space, the pairwise genetic relationships between sample units are expected to vary linearly with the natural logarithm of the geographic distance. The *Sp* statistic defined by Vekemans and Hardy (2004), which enables comparisons among species independent of the sampling scheme, was calculated as $-\hat{b}_F / (1-\overline{F}_{(1)})$; where $\hat{b}_F$ is the slope of the linear regression of $\overline{F}_{(d)}$ on the natural logarithm of the geographic distance, and $\overline{F}_{(1)}$ is the mean F_{ij} over the first distance class. In this formula, the first distance class is supposed to contain all (nearest) neighbour pairs. Since 98% of neighbour pairs of trees were in the 0 – 20 m distance class, the distance limits were set to 20, 30, 40, 50, 100, 200, 300, 400, 500, and 1000 m.

To assess the potential impact of clonal growth form on SGS, a preliminary analysis was performed at the tree level, in which all ramets within a MLL were assigned the same genotype**;** a second analysis was then performed at the MLL level. In the first analysis, the significance of $\overline{F}_{(d)}$ and $\hat{b}_F$ were assessed by 10000-permutation tests based on the geographic locations of trees. In the second analysis, a 10000-resampling approach was used, in which one ramet was randomly selected from each MLL at each resampling step (Alberto et al. 2005). This yielded a 95% confidence interval for $\overline{F}_{(d)}$ for each distance class. The significance of $\hat{b}_F$ was assessed as above.

Results

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Clonality

The genotypic resolution followed an asymptotic trend (Fig. 2**),** where the gain from using additional markers increased sharply between one and four loci and appeared to stabilize at very low values when there were more than six loci (*i.e.*, less than 5% additional MLGs identified per additional locus).

Among the 413 trees, we were able to genotype 379 fully at the 11 SSR loci and these clustered into 222 MLGs. All ramets within a MLG were associated with a p_{sex} value below 10^{-7}. The 34 remaining trees had one (22), two (8), three (3) or four (1) loci missing. By sequentially removing the missing loci before re-analysing the data, it was possible to assign 22 of these trees to previously identified MLGs ($p_{sex} < 10^{-5}$). By sequentially removing the mismatched loci for MLGs differing at only one locus, these MLGs could be clustered into 37 MLLs ($p_{sex} < 10^{-5}$). A total of 194 distinct MLLs were therefore identified, of which 79 $MLL_{\geq 2}$. The resulting genotypic richness (R) was 0.47. Sex data were consistent with this grouping since all ramets within a MLL were of the same gender.

MLL size (N_R) ranged from one to 18 ramets, but 95 % of MLLs contained fewer than five ramets (Fig. 3). The logarithm of the cumulative distribution of ramets among MLLs was significantly linearly related to the logarithm of N_R (Fig. 3), thus supporting the Pareto distribution hypothesis. The associated patchiness exponent estimate was $\beta = 1.07$.

Clonality appeared to be evenly distributed through the study site since differential plotting of individuals belonging to unreplicated genotypes, to small MLLs

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and to large MLLs did not reveal any structured geographical pattern (Fig. 4). MLL geographic size, as measured by the maximum distance between two ramets (d_{max}), ranged from 0.9 to 30.3 m. The intra-MLL average distance between nearest neighbours ($\overline{d}_{neighb}$) ranged from 0.9 to 18.6 m. A significant linear relationship was found between N_R and d_{max} (Fig. 5). Although resulting in a non significant linear correlation coefficient, a triangular relationship was found between N_R and $\overline{d}_{neighb}$: MLLs with few ramets were associated with a large range of $\overline{d}_{neighb}$ values while low $\overline{d}_{neighb}$ values (≤ 5 m) were consistently found in MLLs containing six or more ramets (Fig. 5). A similar relationship was found between N_R and mean or individual tree girth (single-stemmed individuals only): high ramet numbers were associated with low girths while MLL sizes ranging from one to five were associated with a large range of girth values (Fig. 6).

The estimated aggregation index (A_c) was 0.62 ($P < 0.001$), indicating significant spatial clustering of clonal ramets compared to the whole population. The mean clonal dominance index ($\overline{D}_c$) was 0.99, indicating that the spatial range of a MLL was almost exclusively occupied by ramets belonging to that MLL. This parameter differed from 1 in only two $MLL_{\geq 3}$ (D_c = 0.67 and 0.71).

Introgression from the Lombardy poplar

Of the thirteen Lombardy poplars sampled, eleven were similar to the San Giorgio reference genotype at all studied loci. Although belonging to the main group of eleven trees forming a row on a campsite nearby, the two others differed from the San Giorgio genotype at one and two loci, respectively. These differences always corresponded to

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one-repeat-unit changes and were restricted to one allele per differing locus. Genotyping newly collected leaves led to the same results, suggesting somatic mutations had occurred before planting.

Five $MLL_{=1}$ (two males and three females, girth = 41, 159, 164, 189, and 208 cm) were identified as possible F_1 hybrids of the San Giorgio genotype. Some alleles from this genotype were found at very low frequencies in the MLLs (*e.g.*, $f = 0.008$ at locus PMGC2852), thus resulting in low probabilities of false paternity assignment ($7.7 \times 10^{-5} \leq P_{non\text{-}excl.} \leq 2.7 \times 10^{-3}$). None of the identified introgressed hybrids exhibited the typical columnar shape of the Lombardy poplar. Core-analysis of three of them revealed ages of 12.5, 53, and 45.5 years (girth = 41, 159, and 208 cm, respectively). All five individuals were removed from subsequent analyses. No potential F_1 progeny from any of the two identified somaclonal mutants of San Giorgio was found.

Genetic diversity

The sex-ratio was 1 : 0.92 on an individual tree basis, which was not significantly different from a 1 : 1 ratio (Table 1). As no significant difference was found between males and females for both the number of $MLL_{\geq 2}$ (40 and 39, respectively) and the mean number of ramets per $MLL_{\geq 2}$ (i.e., $N_R = 4.1$ and 3.4, respectively, $P = 0.50$), the sex-ratio was also balanced at the MLL level (Table 1).

The level of polymorphism was highly variable among the 11 studied loci, ranging from four (PMGC333) to 22 alleles (PMGC667). High rates of rare alleles led to a two-fold difference between mean observed and effective allele numbers, $\overline{A}$ and

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$\overline{A}_e$ (Table 2). Compared to the nine other loci, PMGC433 and PMGC2838 combined low polymorphism, high rates of rare alleles, and (possibly as a consequence) lower observed and expected heterozygosities, H_o and H_e. Mean $\overline{H}_o$ and $\overline{H}_e$ values were very close, leading to a non-significant overall F_{is} (Table 2). Two loci (PMGC2852 and PMGC333) exhibited significant heterozygote excess and three (PMGC667, PMGC2838 and WPMS05) significant deficit (Table 2).

Spatial genetic structure

At the tree level, the regression of F_{ij} over the natural logarithm of the geographic distance produced a significantly negative regression slope ($\hat{b}_F$ = - 0.0263, $P < 0.001$), indicating higher genetic similarity among trees that were closer together. A significant positive mean kinship coefficient was found in the first distance class only (d_1 = 0 – 20 m, $\overline{F}_{(1)}$ = 0.1870; Fig. 7). At the MLL level, the kinship – distance regression slope was much shallower, but still significant ($\hat{b}_F$ = - 0.0045, P = 0.001; Fig. 7). The *Sp* statistic was seven-fold smaller at the MLL level than at the tree level, decreasing from 0.0324 to 0.0046, while $\overline{F}_{(1)}$ decreased to 0.0230.

Discussion

SSRs have proved efficient in poplars for fingerprinting and for detecting introgression from different species (Fossati et al. 2003; Liesebach et al. 2010; Smulders et al. 2008a). Despite the fact that it belongs to the *P. nigra* species, the

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Lombardy poplar (*i.e.*, the San Giorgio reference genotype) carried some alleles that were comparatively rare in the studied *P. nigra* population. This allowed us to consider 2.6% of MLLs being probable F_1 hybrids of this cultivar with low probabilities of false paternity assignment. Of course, these probabilities are based on the hypothesis that the allelic frequencies observed within the studied population are representative of the population's parental gene pool. However, poplar seeds are dispersed by water over long distances, and Lombardy poplars are very frequent in rural and urban landscapes of the Loire Valley. It is thus expected that introgression events, if any, would most probably originate from crosses upstream of the study site. This idea is supported by age inconsistencies between the studied Lombardy poplar trees and most of the probable introgressed F_1 individuals, and also by the fact that the two Lombardy poplar somaclonal mutants found at close vicinity of the study site were not found to be potential parents of any studied tree. When trying to identify introgression events from the Lombardy poplar in natural *P. nigra* stands, Imbert and Lefèvre (2003) also reported rare alleles at one SSR locus but only mentioned a rough estimate of a few percent introgressed genotypes. Other studies have reported introgression levels between 0% (Tabbener and Cottrell 2003), and 1.6% (Vanden Broeck et al. 2004). Both these studies concluded that there was a negligible threat to local black poplar populations because of the early flowering of the Lombardy poplar, and a consequent lack of synchronism with *P. nigra* females of northern origin. We do not share this optimistic point of view for two main reasons, namely (i) an underestimation of introgression rates due to the fact that advanced-generation intraspecific hybrids cannot be detected with high levels of confidence and (ii) weak support for the asynchronism hypothesis in a species with a wide distribution area, especially in the context of a changing climate. Moreover, the

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five probable identified introgressed individuals were not recognizable based on their phenotype with respect to branching. We thus suspect that genotyping existing *ex-situ* collections of *P. nigra* to check for possible introgression from Italica would produce surprising results.

In previous studies, SSR analysis of commercial cultivars from different taxa (Fossati et al. 2003; Liesebach et al. 2010) and natural *P. nigra* stands (Barsoum et al. 2004; Pospiskova and Bartakova 2004; Pospiskova and Salkova 2006; Rathmacher et al. 2010; Smulders et al. 2008b) allowed detection of replicated genotypes. When considering the evolution of marginal gain in terms of additional differentiated MLGs per additional locus, the 11 SSRs used in the present study allowed a genotypic resolution close to optimum. Indeed, although 'clonality is merely a genotype resolution phenomenon dependent upon the resolution power of molecular markers culminating with direct sequencing of DNA' (Lushai and Loxdale 2002), increasing the number of markers not only allows the detection of rare somatic mutation events but also increases the chance of scoring errors occurring. Somatic mutations are expected to occur at significant rates for SSRs, for which high mutation rates ranging from 10^{-7} to 10^{-3} per locus per generation have been reported in eukaryotes (Buschiazzo and Gemmell 2006). As an illustration, two somatic mutants were identified among the thirteen Lombardy poplars analysed here. However, using the standardized procedure proposed by Arnaud-Haond et al. (2007), MLGs differing at only one locus were grouped into MLLs despite their somatic-mutant *vs.* scoring-error status. Reviewing the data, it appears that there are only four circumstances out of 45 for which a mutational event corresponding to the Stepwise Mutation Model could be hypothesized **(***i.e.*, both MLGs heterozygous with a one-repeat allelic difference). Somatic mutations may be useful tools, acting as a

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molecular clock in many clonal cells or organisms including poplars (Ally et al. 2008; Mock et al. 2008). Nevertheless, there are many pitfalls in their analysis including (i) a lack of knowledge about mutation rates during mitosis, (ii) a complex heterogeneity of mutational events at allele, locus, individual and/or taxon levels, and, again, (iii) the difficulty in distinguishing between true somatic mutations and scoring errors (Heinze and Fussi 2008).

The population studied exhibited substantial asexual recruitment. If one ramet per MLL represented a potential founder, then 53% of the population originated from vegetative propagation. The genotypic richness ($R = 0.47$) was intermediate within the range of values found in other *P. nigra* studies (or computed from them when not originally expressed as $G - 1 / N - 1$). Considering clumped trees to be clonal ramets, as suggested by Barsoum et al. (2004), would lead to even lower genotypic richness values. *R* values across all studied European *P. nigra* stands found in the literature appear to follow a distribution skewed towards higher values, with fifteen values out of nineteen falling between 0.8 and 1 and only three occurrences below 0.2 (Arens et al. 1998; Barsoum et al. 2004; Legionnet 1997; Pospiskova and Bartakova 2004; Pospiskova and Salkova 2006; Rathmacher et al. 2010; Smulders et al. 2008b). Very low values of 0.01 and 0.04 have also been reported in mature stands in Great Britain (Smulders et al. 2008b) and in the Netherlands (Arens et al. 1998), respectively, although both sampling schemes were designed to avoid collecting clonal individuals. In contrast, despite a nearest neighbour sampling strategy, Barsoum et al. (2004) found high *R* values (> 0.8) in three age cohorts, with a significantly higher number of clonal ramets in the 'middle-aged' stands (8 years old) than in both the 'young' (5.6 years old) and 'old' (17.6 years old) stands. Sampling in this previous study covered islands and

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gravel bars, each of them having certainly been more favourable (spatially and temporally) for seedling recruitment and also less affected by anthropogenic disturbance than our study site. Tree densities were consistently higher on the islands and gravel bars than those recorded in Saint-Ay (0.2 trees.m^{-2} in 'old' stands *vs.* 0.006 trees.m^{-2} in the current study), and the existence of more dynamic sites certainly explained why the 'old' cohorts encountered by Barsoum et al. (2004) were much younger than most individuals examined by us. Vegetative propagation in Saint-Ay certainly benefited from the availability of open space, although the sites available for colonization were generally unfavourable (spatially and temporally) for seedling recruitment.

The MLL size (N_R) distribution was skewed towards smaller values, ranging from one to 18 ramets and exhibiting exponential decay. In previous studies, only small clones of two to four ramets have been observed (Barsoum et al. 2004; Legionnet 1997; Rathmacher et al. 2010) while Arens et al. (1998) and Smulders et al. (2008b) found larger clones of up to 22 and 32 ramets, respectively. A clone size of 70 ramets was recently reported in a British population, but this was probably planted (Smulders et al. 2008b). The β Pareto index associated with the partitioning of ramets among MLL size classes should allow reliable comparisons between studies. The present study provides a first estimate of β in *P. nigra*. The calculated value (1.07) was moderate in comparison with those presented in a literature review pertaining to several clonal species (Arnaud-Haond et al. 2007). These authors reported extreme values of 0.06 (*Posidonia oceanica*) and 2.96 (*Sinularia flexibilis*), indicating dominance of some large clonal patches and high evenness, respectively. They also provided the only reference available for a tree species, namely *Prunus ssiori* (β = 0.88).

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Considering within-clone, between-clone, and between-species contacts, Lovett Doust (1981) recognized a spectrum of growth forms in clonal plants, with the two extremes referred to as 'phalanx' and 'guerrilla' forms. The high aggregation (A_c = 0.62) and clonal dominance (D_c = 0.99) indexes computed in the present study allow us to conclude that *P. nigra* exhibits a typical 'phalanx' growth form, where ramets of the same MLL are aggregated and do not share their space with ramets of any other MLL. Despite being less explicit, all published data on *P. nigra* clonal growth are also indicative of a 'phalanx' growth form with zero or near-zero intermingling of clones (Barsoum et al. 2004; Legionnet 1997). Although only possible with non-exhaustive sampling strategies, larger study areas have allowed the identification of long distance dispersal events up to 19 km (Barsoum et al. 2004), while the maximum distance found in the current study was d_{max} = 30.3 m. The significant positive correlation between N_R and d_{max} and the absence of a significant correlation between N_R and $\overline{d}_{neighb.}$ may indicate that clonal growth in this open habitat is an expansion process rather than one that leads to a densification of clonal patches. The triangular relationships found between N_R and both $\overline{d}_{neighb.}$ and girth need further examination, however. The fact that the MLLs with high ramet numbers comprised small trees growing close together could be the result of either poor-quality, stressful, micro-habitat conditions promoting vegetative propagation, or a possible genotypic trade-off between the number and size of ramets, as found in other clonal species (Stuefer et al. 2002), Although the whole study site appeared to be favourable for clonal propagation, and although a significant correlation was found between girth and age, the first hypothesis cannot be rejected. More precise tree ages and thus, more detail pertaining to intra-MLL age structure, would facilitate investigations and interpretations. Core analysis is,

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however, very difficult in *P. nigra* wood, as experienced here, and root age would certainly be more informative than stem age when studying clonal growth. It has been hypothesized that flood training is a key mechanism of asexual regeneration in *P. nigra* (Barsoum et al. 2004), but we did not observe the linear ramet distributions associated with this type of sprouting frequently at the study site. Although no excavation was conducted, root suckering seems the most probable type of vegetative spread on this site. The aggregated pattern could thus result from the emergence of new shoots from the parental root system and be maintained by the selective advantage of permanent or at least transient physiological integration (*i.e.,* physical links between ramets) over fragmentation, as expected in habitats with restricted favourable patches compared to unfavourable ones (Oborny and Kun 2002). However, inferring the temporal dynamics of clonal growth from spatial structure at a single time point can be problematic for three reasons: (i) the difficulty of disentangling the timing of the colonization process from density-dependent events (*e.g.,* both recent colonization in an empty space and competitive exclusion can result in a segregated distribution); (ii) there are possible trade-offs between clonal growth forms of a given species under different environmental conditions (Ye et al. 2006); and (iii) community-level analysis, including among-species interactions, is required (Gough et al. 2002).

SSR-based observed and expected heterozygosities found in the literature for *P. nigra* vary within the ranges 0.67 – 0.93 and 0.65 – 0.90, respectively (Fossati et al. 2003; Imbert and Lefèvre 2003; Pospiskova and Bartakova 2004; Pospiskova and Salkova 2006; Rathmacher et al. 2010; Smulders et al. 2008b; Storme et al. 2004; Van Dam and Bordacs 2002). The values reported here ($\overline{H}_o = 0.68$, $\overline{H}_e = 0.69$) are very close to the lower limits. Overall, the value of F_{is} (0.008, n.s.) did not indicate any

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significant deviation from Hardy-Weinberg equilibrium. Since no significant difference was found between male and female vegetative propagation potentials, the sex-ratios were equally balanced at both the tree and MLL levels. We are not aware of any previously published data on the relative vegetative propagation potential of the two genders of *P. nigra*.

Clonality was the main driver of SGS in the studied population. In total, 90% of the identified $MLL_{\geq 2}$ exhibited a d_{max} falling within the distance range of significant kinship coefficients (F_{ij}) found at the tree level (0 – 20 m). Both the slope ($\hat{b}_F$) of the linear regression of F_{ij} over the natural logarithm of geographic distance and the associated *Sp* statistic sharply decreased at the MLL level. The presence of significant residual SGS at the MLL level is consistent with two recent reports relating to *P. nigra* (Pospiskova and Salkova 2006; Rathmacher et al. 2010). Although both studies excluded clonal ramets from the analysis, they reported higher values for both *Sp* (0.0166 and 0.0146 *vs.* 0.0046 in the present study) and $\hat{b}_F$ (-0.0158 and -0.0136 *vs.* -0.0045). The scale of these previous studies was, however, much larger (5 km and 2.5 km, respectively), possibly leading to a sub-structuring of populations as suggested by significant positive overall F_{is} values (*i.e.*, the Wahlund effect). When calculating *Sp* statistics for 47 plant species, Vekemans and Hardy (2004) found values ranging from 0.0003 to 0.2632. They pointed out that the breeding system, life form (*i.e.*, herbaceous, small trees or trees), and population density were statistically linked to patterns of SGS. When considered in isolation, pollen and seed-dispersal modes were not found to be good predictors. Epperson (2007) expected unbalanced seeds *vs.* pollen dispersal patterns to generate SGS, but experimental and theoretical data do not fully support such a general trend (Ng et al. 2006; Sagnard et al. 2010). Results found in the literature

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from paternity (pollen) and parent-pair (seeds) assignments in *P. nigra* are scarce and highly variable. Pospiskova and Salkova (2006) reported maximum distances for pollen and seed dispersal of 230 and 370 m, respectively. Rathmacher et al. (2010) found that 50% of the pollen and 30% of the seeds of the species travelled more than 500 m.

In- and *ex-situ* conservation can both benefit from a better description of clonality. Past samplings in natural *P. nigra* stands across Europe for *ex-situ* conservation have yielded a gene bank collection with 26% duplicated accessions (Storme et al. 2004). Although some of the populations studied were probably composed of vegetative copies propagated by humans and distributed over large areas through cuttings, inappropriate sampling schemes certainly also contributed to this result. Most studies on black poplar, including this one, reported a 'phalanx' growth form usually with small numbers of ramets per clone. Consequently, duplications could be minimized by using an appropriate sampling mesh in combination with sex determination whenever possible. However, the deployment of high-throughput molecular techniques would allow efficient detection of clones at limited cost: as few as six SSR markers proved sufficient to identify 95% of the MLGs in the present study. With respect to *in-situ* conservation, clone size (N_R) distribution may be a critical factor to take into account since the larger the number of ramets, the longer the clone may survive under a gap disturbance regime, as has been simulated for *P. tremuloides* (Namroud et al. 2006). However, these simulations were not spatially explicit. At the species – rather than genotype – level, clonal growth form has been shown to affect competitiveness in a plant community, with ramet aggregation reducing the competitive ability of a clonal species in an open environment (Lenssen et al. 2005). Clustering can also affect mating patterns in dioecious species (Charpentier 2002). In addition, possible

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trade-offs between sexual and asexual fecundities may occur, as documented for other species (Sun et al. 2001) with different implications at the tree, clone or population levels.

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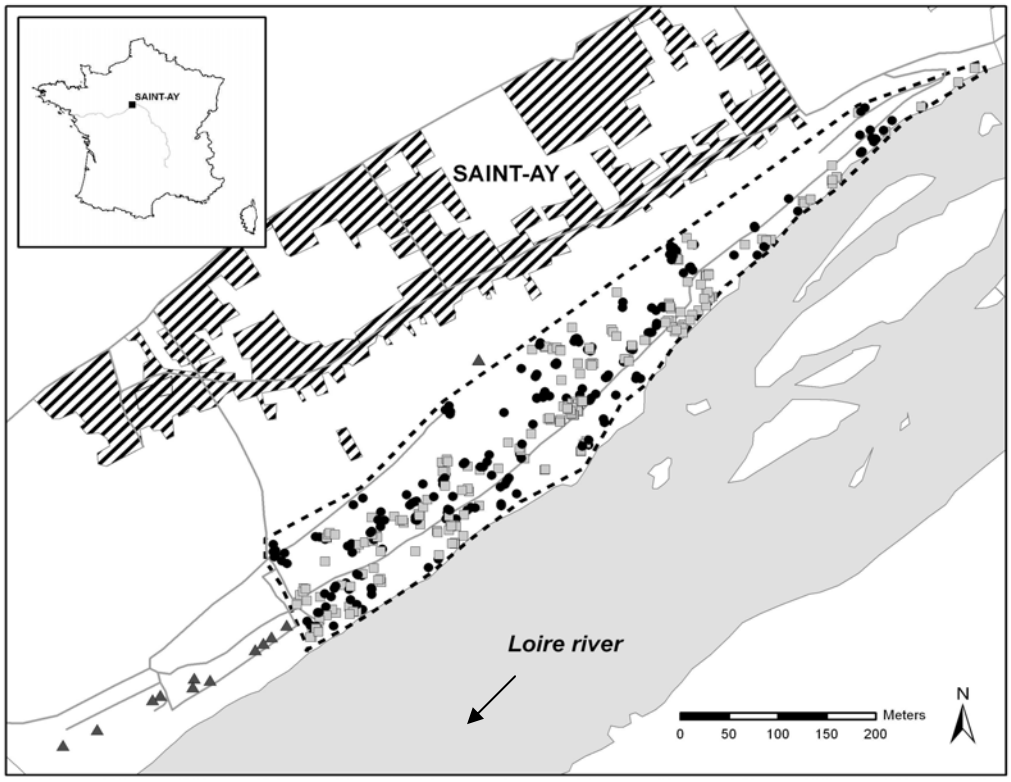


Fig. 1 – Study site (dotted line). Exhaustive inventory of adult trees within this 7 ha area revealed 199 female (black circles) and 214 male (grey squares) wild *P. nigra* trees. Triangles refer to the 13 sampled Lombardy poplars (non exhaustive inventory) used for genotyping and paternity analysis.

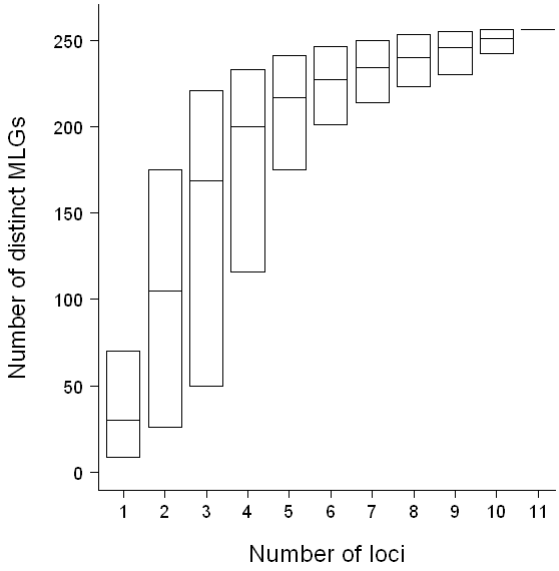


Fig. 2 – Genotypic resolution associated with each possible SSR combination. The boxes are bounded by the most and least informative combinations of loci. The inner line represents the mean value.

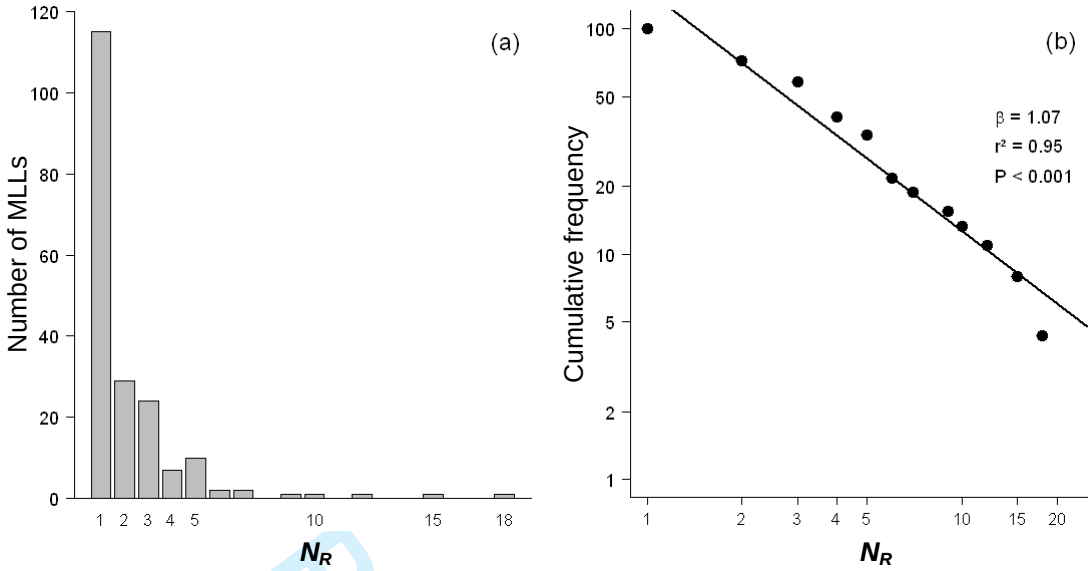


Fig. 3 – (a) Distribution of MLL size classes (N_R, number of ramets) and (b) associated log-log reverse cumulative frequency distribution.

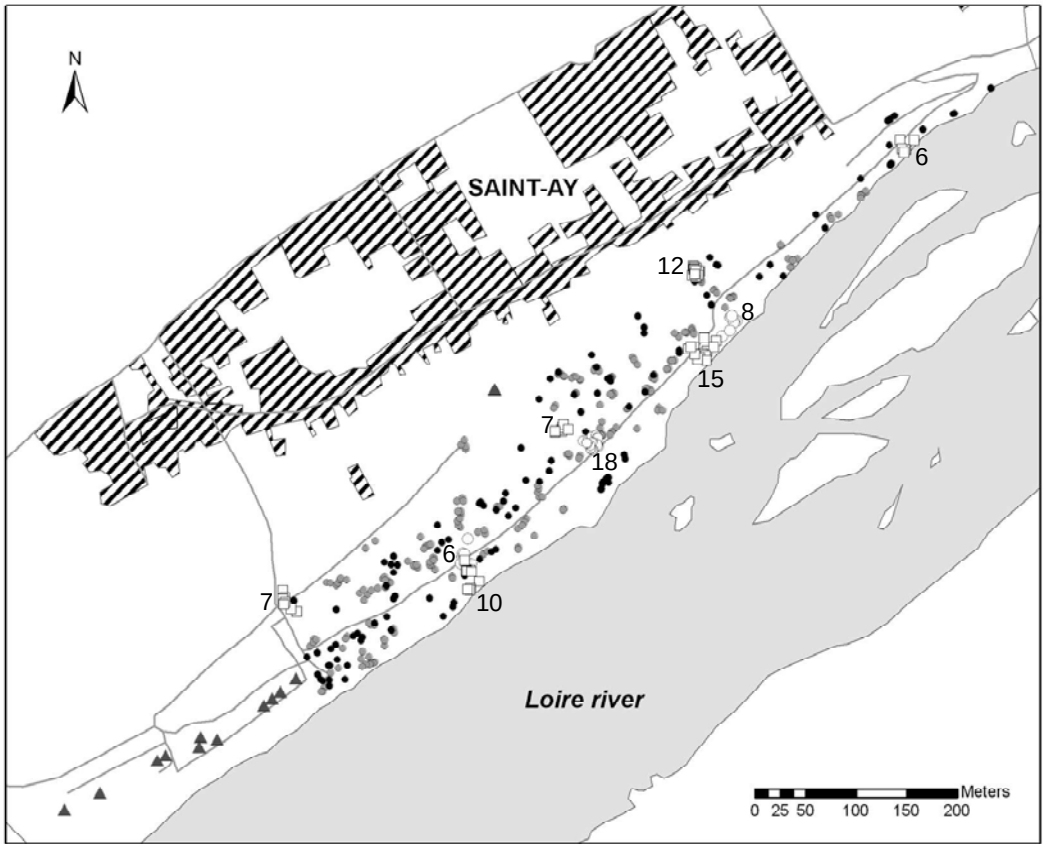


Fig. 4 – Differential plotting of studied individuals belonging to an $MLL_{=1}$ (*i.e.*, unreplicated individuals ; black dots), an $MLL_{2\leq N_R\leq 5}$ (grey dots), or an $MLL_{\geq 6}$ (white dots and white squares with numbers indicating the corresponding ramet numbers). Triangles refer to the 13 sampled Lombardy poplars.

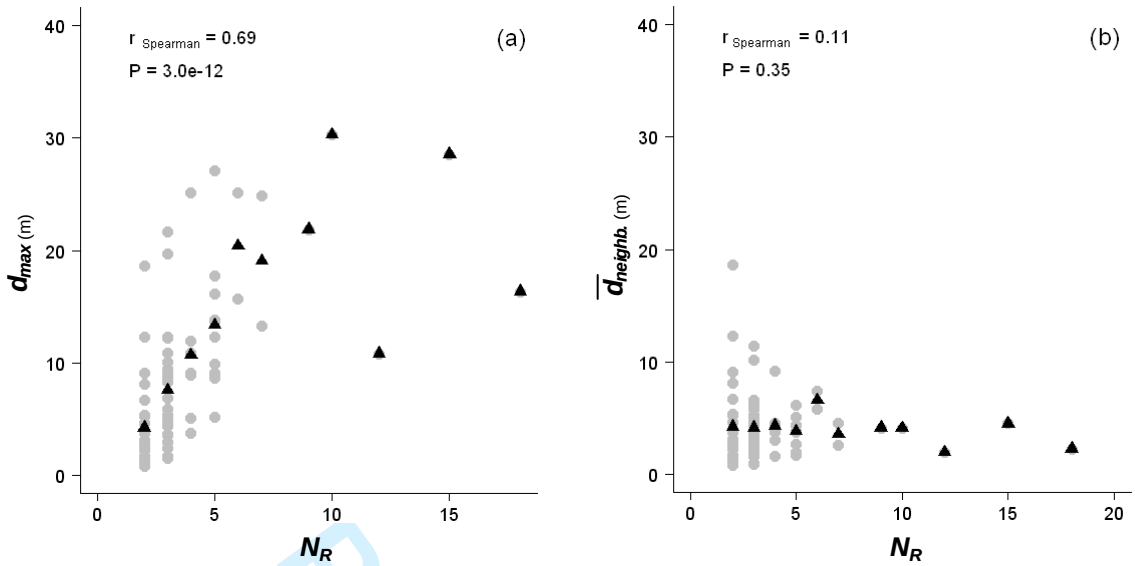


Fig. 5 – Relationships between MLL size (N_R, number of ramets) and (a) the maximum distance between two ramets (d_{max}), (b) the mean distance between closest neighbours ($\overline{d}_{neighb.}$). Mean values (black triangles) were computed for each N_R class. Spearman's correlation coefficients were computed at the MLL level.

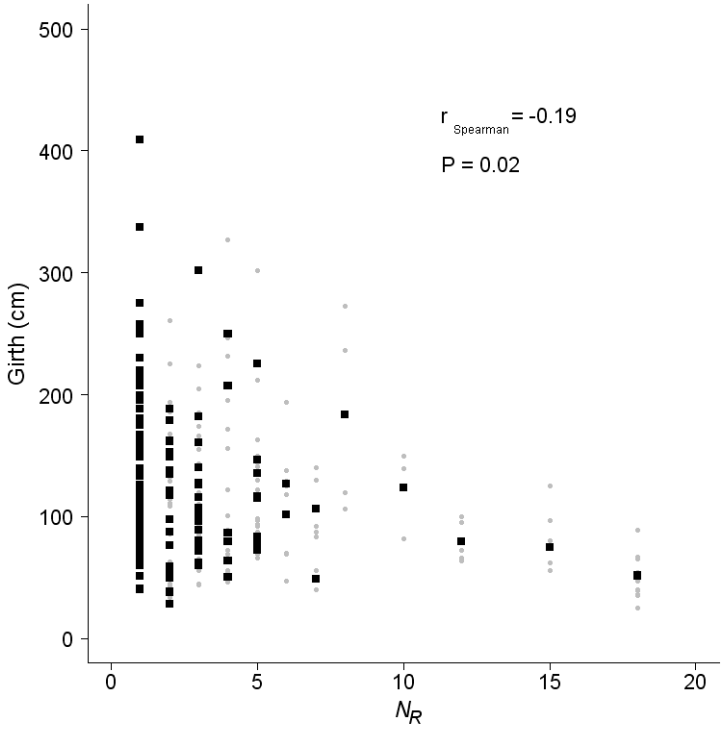


Fig. 6 – Relationships between MLL size (N_R, number of ramets) and girth at breast height at the individual ramet level (grey dots) and at the MLL mean level (black squares). Analysis was restricted to single-stemmed individuals. Spearman's correlation coefficient was computed for the MLL mean level.

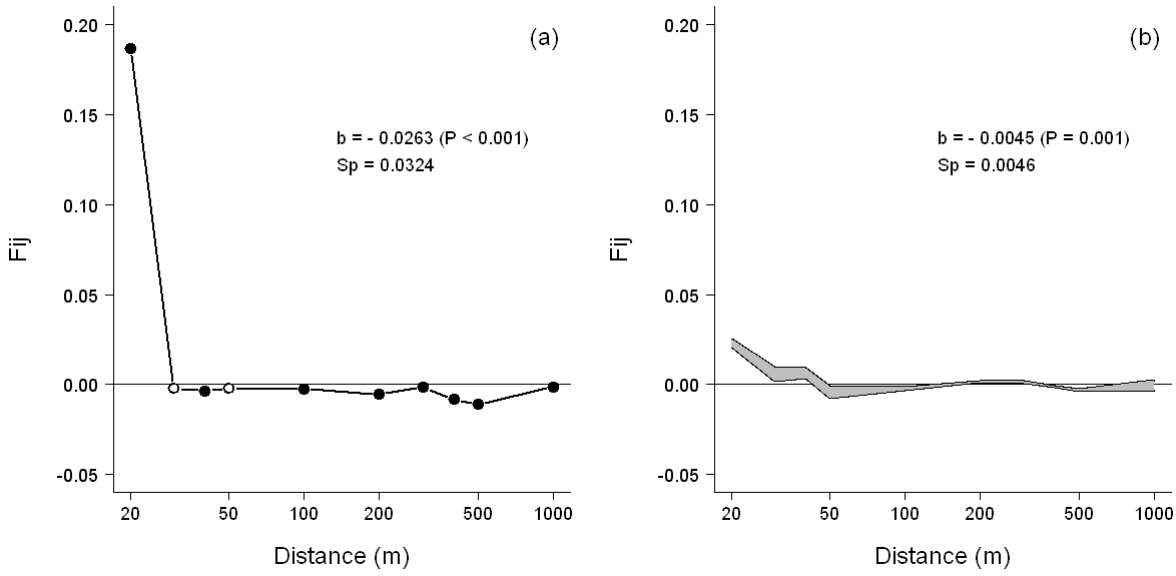


Fig. 7 – Spatial genetic structure analysis at (a) tree and (b) MLL levels. Both correlograms show the evolution of mean kinship coefficients (F_{ij}) between pairs of sampling units over ten geographic distance classes. At the tree level, significant ($P < 0.05$) and non-significant mean F_{ij} values are represented by black and white circles, respectively. At the MLL level, the envelope (95% CI) is the result of a 10000-resampling procedure (a single ramet selected in each MLL at each resampling step). The five trees identified as probable F_1 siblings originating from the Lombardy poplar were removed from the analysis.

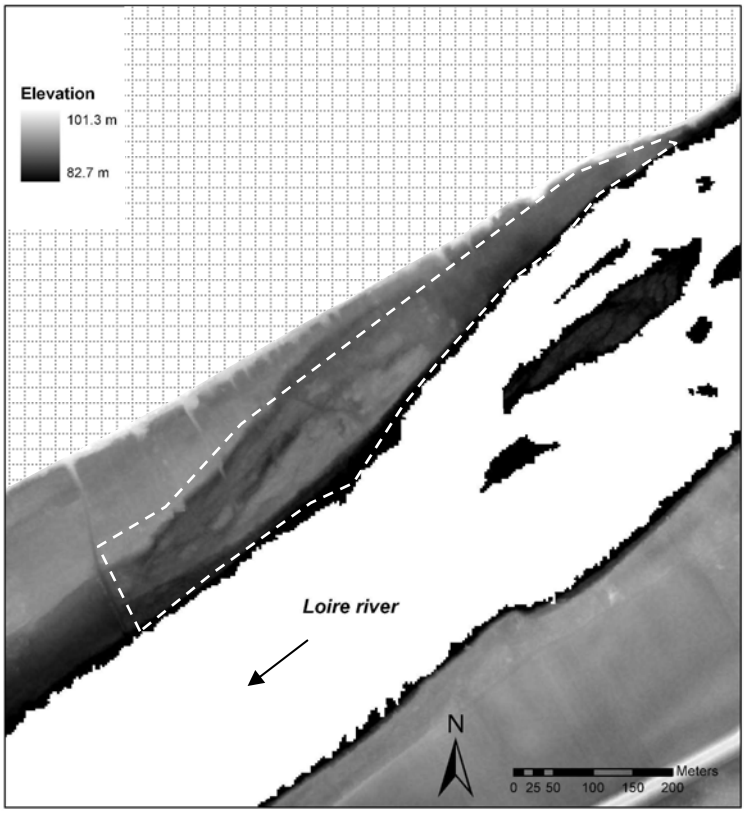
Table 1 – Sex-ratio at tree and MLL levels with a distinction between mono-ramet ($MLL_{=1}$) and multi-ramet ($MLL_{\geq 2}$) MLLs. The five trees identified as probable F_1 siblings originating from the Lombardy poplar were removed from the analysis.

	Number of trees	Number of MLLs		
		$MLL_{=1}$	$MLL_{\geq 2}$	Total
Males	212	48	40	88
Females	196	62	39	101
Total	408	110	79	189
Sex-ratio	1 : 0.92	1 : 1.29	1 : 0.98	1 : 1.15
$P(>\chi^2_{1:1})$	*0.43*	*0.18*	*0.91*	*0.34*

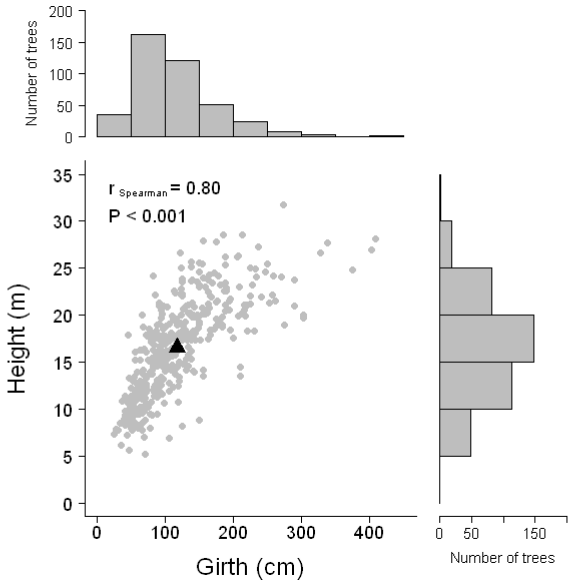
Table 2 – Genetic diversity at the MLL level. The five trees identified as probable F_1 siblings originating from the Lombardy poplar were removed from the analysis.

Locus	LG	Motif	*A*	A_e	H_o	H_e	F_{is} [a]
PMGC2852	I	$(GA)_n$	13	5.4	0.91	0.82	- 0.12***
PMGC667	II	$(GA)_n$	22	9.1	0.73	0.89	0.19***
PMGC486	III	$(GA)_n$	10	5.3	0.85	0.81	- 0.05
PMGC2235	IV	$(GA)_n$	13	3.9	0.73	0.75	0.02
PMGC2838	V	$(GA)_n$	5	1.6	0.37	0.38	0.03**
PMGC2578	VI	$(GA)_n$	15	4.3	0.74	0.77	0.04
PMGC61	VIII	$(CTT)_n$	7	4.3	0.74	0.77	0.04
PMGC333	XI	$(CTT)_n$	4	2.7	0.72	0.64	- 0.14**
WPMS05	XII	$(GT)_n$	14	6.8	0.83	0.86	0.03*
PMGC14	XIII	$(GA)_n$	7	3.9	0.76	0.75	- 0.01
PMGC433	XVI	$(GA)_n$	6	1.2	0.15	0.16	0.08
Overall [b]			10.5	4.4	0.68	0.69	0.008
± SD			± 5.4	± 2.3	± 0.23	± 0.22	

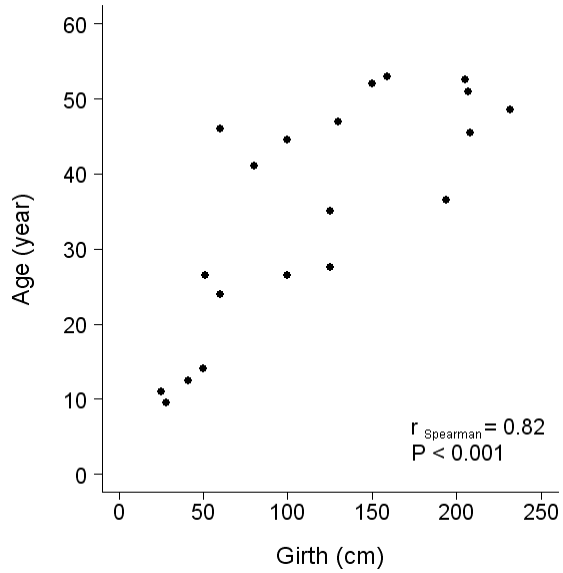
[a] Significant deviation from Hardy-Weinberg equilibrium : **P*<0.05; ***P*<0.01; ****P*<0.001
[b] Mean value (*A*, A_e, H_o, and H_e) or global sample estimate (F_{is})



Supplementary Material 1 – Study site (dotted line) aircraft laser altimetry (data from Direction Régionale de l'Environnement de l'Aménagement et du Logement, Service Loire et Bassin Loire Bretagne, Orléans, France, 2002).



Supplementary Material 2 – Relationship between tree height and girth at breast height and relative distributions of both traits within the studied wild *P. nigra* population (413 trees). The triangle refers to the mean point. In the case of clumped or forked trees, the girth of each stem was measured and the largest value was recorded.



Supplementary Material 3 – Relationship between girth at breast height and tree age estimates (core analysis) on a subset (20 single-stemmed trees) of the studied *P. nigra* population. Increment core samples could not be collected from trees with a girth greater than 250 cm.