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Research paper

Genetic variation for leaf morphology, leaf structure and leaf carbon isotope discrimination in European populations of black poplar (*Populus nigra* L.)

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To buffer against the high spatial and temporal heterogeneity of the riparian habitat, riparian tree species, such as black poplar (*Populus nigra* L.), may display a high level of genetic variation and phenotypic plasticity for functional traits. Using a multisite common garden experiment, we estimated the relative contribution of genetic and environmental effects on the phenotypic variation expressed for individual leaf area, leaf shape, leaf structure and leaf carbon isotope discrimination ($\Delta^{13}C$) in natural populations of black poplar. Twenty-four to 62 genotypes were sampled in nine metapopulations covering a latitudinal range from 48°N to 42°N in France and in Italy and grown in two common gardens at Orléans (ORL) and at Savigliano (SAV). In the two common gardens, substantial genetic variation was expressed for leaf traits within all metapopulations, but its expression was modulated by the environment, as attested by the genotype × environment ($G \times E$) interaction variance being comparable to or even greater than genetic effects. For LA, $G \times E$ interactions were explained by both changes in genotype ranking between common gardens and increased variation in SAV, while these interactions were mainly attributed to changes in genotype ranking for $\Delta^{13}C$. The nine *P. nigra* metapopulations were highly differentiated for LA, as attested by the high coefficient of genetic differentiation ($Q_{ST} = 0.50$ at ORL and 0.51 at SAV), and the pattern of metapopulation differentiation was highly conserved between the two common gardens. In contrast, they were moderately differentiated for $\Delta^{13}C$ ($Q_{ST} = 0.24$ at ORL and 0.25 at SAV) and the metapopulation clustering changed significantly between common gardens. Our results evidenced that the nine *P. nigra* metapopulations present substantial genetic variation and phenotypic plasticity for leaf traits, which both represent potentially significant determinants of populations' capacities to respond, on a short-term basis and over generations, to environmental variations.

Keywords: bulk leaf carbon isotope discrimination, common gardens, leaf traits, natural populations, phenotypic plasticity, population differentiation.

Introduction

Forest tree species generally have a broad geographical distribution encompassing a wide range of environmental conditions and large effective population sizes (Petit and Hampe 2006). Both of these characteristics are supposed to favour the maintenance of a high level of genetic variation for functional traits, such as bud phenology, growth rate or water-use efficiency (WUE) (Alberto et al. 2013). At the leaf level, leaf structure, leaf area and leaf shape vary tremendously between and within species as well as in response to environmental conditions, and these variations are

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known to play a key role in shaping plant functioning (Wright et al. 2004, 2005, Niinemets and Sack 2006, Reich et al. 2007, Nicotra et al. 2011, Niinemets 2015). Over generations and at the population level, a high amount of intraspecific genetic variation may offer possible adaptive responses of populations to directional selection involving these functional traits (Jump et al. 2009). On a short-term basis and at the whole-organism level, phenotypic plasticity, i.e., the ability of a genotype to produce different phenotypes depending on environmental conditions, is also regarded as a major way by which trees could withstand environmental heterogeneity (Bradshaw 1965). An enhanced knowledge of the interplay between genetic variation and phenotypic flexibility in responses of natural populations to environmental changes may increase our ability to develop informed management plans for in situ or ex situ conservation of endangered genetic resources (Forsman 2014).

Located at the interface between aquatic and terrestrial ecosystems, riparian zones are characterized by a high degree of spatial and temporal heterogeneity (Karrenberg et al. 2002) that may promote the maintenance of a high level of phenotypic plasticity and genetic variation in riparian tree species. Black poplar (*Populus nigra* L.) is one of the dominant pioneer tree species of riparian ecosystems in the temperate climatic zones of Europe, Northern Africa and Western Asia (Dickmann and Kuzovkina 2008). Pollen and seeds of black poplar are dispersed by wind and to a lesser extent by water over several kilometres. Thus, gene flow is likely to occur over long distances within river catchments and *P. nigra* populations generally form metapopulations, i.e., a network of local populations interconnected by significant gene flow (Lefèvre et al. 2001). Evaluating the expressed genetic variation and phenotypic plasticity for traits influencing plant functioning in different environmental conditions may help to gain insight into the geographical structure of phenotypic variation and the ability of natural populations to cope with environmental variations.

The phenotypic plasticity for leaf dimensions, specific leaf area (SLA) and leaf-level WUE (generally evaluated through bulk leaf carbon isotope discrimination, $\Delta^{13}C$), has been well documented in hybrid poplars. These traits have been found to be modulated by various environmental factors, including light (Larocque 1999, Casella and Ceulemans 2002, Al Afas et al. 2005, Benomar et al. 2011, Toillon et al. 2013), nutrient availability (Ibrahim et al. 1997, Siegwolf et al. 2001, Cooke et al. 2005, DesRochers et al. 2006, 2007, Toillon et al. 2013) and water availability (Liu and Dickmann 1992, Ibrahim et al. 1997, Marron et al. 2003, Monclus et al. 2006, 2009, DesRochers et al. 2007, Toillon et al. 2013). In this regard, natural populations of pure poplar species have received much less attention (Zhang et al. 2004, Xu et al. 2008, Chamaillard et al. 2011). In other forest tree species, the phenotypic plasticity of leaf dimensions, SLA and $\Delta^{13}C$ has been widely documented and further confirmed the high plasticity of these traits in response to variations in light (e.g., Valladares et al. 2000, Aranda et al. 2007), nutrient availability (e.g., Curtis et al. 1995, Valladares et al. 2000, Ripullone et al. 2004) and water availability (e.g., Aranda et al. 2007, 2010, Corcuera et al. 2010, de Miguel et al. 2012).

Besides phenotypic plasticity, significant phenotypic variation has been reported for leaf area, SLA and $\Delta^{13}C$ among genotypes of different hybrid poplars (e.g., Marron et al. 2005, Monclus et al. 2005, Marron and Ceulemans 2006, Bonhomme et al. 2008, Toillon et al. 2013) as well as among genotypes within natural populations of different pure poplar species (Dunlap et al. 1995, Gornall and Guy 2007, Kanaga et al. 2008, Soolanayakanahally et al. 2009, Keller et al. 2011), including black poplar (Chamaillard et al. 2011). However, quantification of population differentiation for these leaf traits in poplar species remains much less documented at a large geographical scale. Only one study conducted on 20 natural populations of balsam poplar (*Populus balsamifera* L.), covering the entire distribution range over North America, evidenced that SLA was highly differentiated among populations, as estimated by the coefficient of genetic differentiation ($Q_{ST} = 0.47$), while $\Delta^{13}C$ was moderately differentiated among populations ($Q_{ST} = 0.21$ for leaf and wood $\Delta^{13}C$). In other forest tree species, several studies have reported significant geographical variation for SLA and $\Delta^{13}C$ (e.g., Zhang et al. 1993, Lauteri et al. 2004, Grossnickle et al. 2005, Ducrey et al. 2008, Aletà et al. 2009). However, only a few reported Q_{ST} estimates (Ramírez-Valiente et al. 2009, Lamy et al. 2011). These values were low to moderate for leaf traits ($0.06 \leq Q_{ST} \leq 0.28$) (Ramírez-Valiente et al. 2009, 2010, Lamy et al. 2011), with the exception of leaf size in *Quercus suber* L. for which natural populations were highly differentiated ($Q_{ST} = 0.46$) according to water availability at the sites of origin (Ramírez-Valiente et al. 2009, 2010). Overall, studies examining the genetic variation expressed for leaf traits in forest tree species have been conducted under single environments. As a consequence, they did not enable testing either for the robustness of population differentiation over environments or the joint contribution of phenotypic plasticity and genetic variation in determining the phenotypic variation.

The aim of the present study was to evaluate the relative contribution of environmental and genetic effects in phenotypic variation expressed for leaf morphology, leaf structure and $\Delta^{13}C$ in natural populations of black poplar to provide a first insight into their ability to cope with environmental variations. Measurements were conducted on 24–62 *P. nigra* genotypes sampled in nine European metapopulations naturally distributed along nine river catchments covering a latitudinal range from 48°N to 42°N and grown in two common gardens in France and in Italy. This two-site common garden experiment allowed us to estimate the relative contribution of genetic, environmental and genotype by environment ($G \times E$) interaction effects on the total phenotypic variation. Thus, the specific objectives of the study were to evaluate: (i) the phenotypic plasticity of genotypes for the different leaf traits and its genetic variation in the *P. nigra* populations and

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(ii) the level and the geographical patterns of genetic variation in the *P. nigra* populations. Additionally, a previous common garden experiment conducted on a set of 14 European *P. nigra* populations partially overlapping with the one studied here reported a strong latitudinal cline for growth cessation and bud set (Rohde et al. 2011). We therefore also checked whether the patterns observed for leaf traits here are comparable to those observed for growth cessation and bud set in this previous study.

Materials and methods

Populus nigra collection and experimental design

This study makes use of a *P. nigra* collection composed of 1098 cloned individuals sampled in 14 natural metapopulations present in 11 river catchments in four European countries. In this study, the term 'metapopulation' refers to a group of individual trees, sexually mature or not, that were sampled in one or several stands distributed along the same river and geographically close enough to be interconnected by gene flow (Lefèvre et al. 2001). This collection was established in common gardens in 2008 at two experimental sites located in Central France (Orléans, ORL, Loire valley, 47°50′N 01°54′E, 108 m above sea level, a.s.l.) and in Northern Italy (Savigliano, SAV, Po valley, 44°36′N 07°37′E, 343 m a.s.l.). The whole *P. nigra* collection was set up in ORL, while a subset of 815 genotypes from the 14 metapopulations was set up in SAV. For each site, plantations were established from 25 cm hardwood cuttings according to a completely randomized block design with a single tree per block and six replicates per genotype. Initial spacings within and between rows of 1.00 × 2.00 m in ORL and 0.80 × 2.20 m in SAV were used. A double border row of the *P. nigra* genotype 'Jean Pourtet' was planted around the experimental plot to reduce border effects. The experimental plantations were regularly irrigated, without fertilizer addition, weed controlled and treated with insecticides as necessary throughout the two growing seasons considered in this study (2008 and 2009).

Meteorological data for each experimental site were obtained from the closest meteorological station (Saint-Cyr-en-Val for ORL; Lagnasco for SAV). In 2009, the growing season of main interest in this study, the range of temperatures was similar between the two experimental sites (see Figure S1 available as Supplementary Data at *Tree Physiology* Online), but annual precipitation was higher at SAV than at ORL (968 vs 665 mm). Soil composition of the upper layers was analysed by collecting spatially distributed soil core samples across the two experimental sites at a 0–40 cm depth. Soil analyses were carried out after planting in both experimental sites. For each site, soil texture (% clay, % silt and % sand), pH and composition of organic matter (OM, g kg^{-1}), organic carbon (C, g kg^{-1}) and total nitrogen (N, g kg^{-1}) were determined. Results indicated that in ORL the soil was a loamy-sandy type (16.2% clay, 15.4% silt and 68.4% sand) while the soil was a loamy type in SAV (21.6% clay, 36.7% silt and 41.7% sand). The soil in SAV was richer in OM (27.9 vs 19.9 g kg^{-1}), C (16.1 vs 11.5 g kg^{-1}) and N (1.4 vs 0.6 g kg^{-1}) and had a higher pH than in ORL (8.0 vs 5.4). The loamy texture and the higher level of soil fertility characterizing SAV induced a substantial increase of tree growth potential when compared with ORL: at the end of the first growing season (2008), circumference at 1 m above ground level ranged from 27 ± 4 to 110 ± 5 mm between extreme genotypic means at SAV vs 17 ± 4 to 31 ± 2 mm at ORL. To prevent mutual shading of trees during the year of study (i.e., 2009), trees were cut back in February 2009 at SAV and only the most vigorous stem of each stool was preserved after resprouting.

For this study, we selected a subset of 308 and 307 genotypes from nine metapopulations in ORL and SAV, respectively; among them, 296 genotypes were common to both common gardens (Table 1). The selected metapopulations are naturally distributed along nine river catchments covering a latitudinal range from 48°N to 42°N in France and in Italy (Table 1). Most of these river catchments are highly dynamic and provide a constant supply of open area where different uneven-aged cohorts are established (Table 1). However, due to the lack of suitable area for spontaneous regeneration, three metapopulations no longer displayed a dynamical behaviour in terms of demography and only consist of old mature trees progressively being replaced by successional hardwood trees (Table 1). No introgression from interspecific cultivated poplar plantations was detected within all selected metapopulations (data from molecular analyses not shown).

Phenotypic data collection

Measurements were carried out in 2009, 1 year after planting. The same traits were measured in the two common gardens but the number of studied trees per genotype differed between common gardens and traits (Table 2).

Dimensions and shape of mature leaves At the end of July 2009, an average of two neoformed mature and fully illuminated leaves (one to six according to their size) were collected on the terminal shoot of each tree in ORL and SAV. A digital picture of each collected leaf was immediately taken after sampling and analysed using LAMINA software (Bylesjö et al. 2008) to determine lamina length, lamina width, lamina perimeter (*P*, cm) and lamina area (LA, cm^2). The ratio between lamina perimeter and lamina area (*P* : LA, cm^{-1}) was then calculated and used as a proxy of lamina shape (Sack et al. 2003). Petiole length was measured using ImageJ software (Abràmoff et al. 2004). All estimated values were averaged to give a single tree value.

Structure and function of mature leaves Three (at SAV) and four (at ORL) calibrated discs (0.80 and 2.0 cm^2, respectively) were punched from each lamina and oven-dried at 60 °C for 72 h before being weighed to compute SLA (cm^2 g_{DM}^{-1}). Carbon isotope composition ($\delta^{13}C$) was measured from 1-mg dry homogenous leaf powder using an elemental analyser (Carlo Erba, Milan,

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Table 1. Location, river management and number of studied genotypes in ORL and SAV for the nine *P. nigra* metapopulations. Where metapopulations were represented by individual trees sampled in different stands distributed along one river, a range of latitudes, longitudes and altitudes is given. Metapopulations were ordered by country according to the latitude of origin. Altitude is expressed in metres a.s.l.

Country	River catchment	Metapopulation	Latitude	Longitude	Altitude	Cohorts[1]	River management[2]	Number of studied genotypes		
								ORL	SAV	Common
France	Rhin	Rhin	48°16′N–48°37′N	07°41′E–07°49′E	135–160	Mature	Regulated	31	32	31
France	Loire	Saint-Pryvé	47°51′N	01°48′E	90	Juvenile/mature	Dynamic	27	26	25
France	Allier	ValAllier	46°24′N	03°19′E	220	Juvenile/mature	Dynamic	30	31	30
France	Dranse	Dranse	46°23′N	06°30′E	374	Juvenile/mature	Dynamic	30	30	26
France	Drôme	Ramières	44°41′N–44°45′N	04°55′E–05°24′E	145	Juvenile/mature	Dynamic	44	43	42
France	Adour	Adour	42°53′N–43°23′N	0°02′W–00°56′W	52–902	Mature	Partially regulated	30	31	30
France	Nohèdes	Nohèdes	42°37′N	02°17′E	820	Mature	Dynamic	28	28	27
Italy	Ticino	Ticino	45°12′N–45°16′N	08°59′E–09°04′E	60–70	Juvenile/mature	Dynamic	62	62	61
Italy	Paglia	Paglia	42°45′N–42°52′N	11°45′E–11° 55′E	235–358	Juvenile/mature	Dynamic	26	24	24

[1]Juvenile trees were defined as non-reproductive trees.
[2]Regulated if water flows have been regulated to facilitate navigation or to prevent floods; dynamic if water flows are not regulated and allow some flooding events.

Table 2. List of traits measured in ORL and SAV common gardens with their abbreviation used in the text, figures and tables and number of studied trees per genotype. DM, dry mass.

Trait	Definition	Unit	Number of studied trees per genotype	
			ORL	SAV
LA	Lamina area	cm^2	5	6
P : LA	Ratio between lamina perimeter and lamina area	cm^{-1}	5	6
SLA	Specific leaf area	$cm^2\ g_{DM}^{-1}$	3	6
N_{mass}	Leaf nitrogen content on leaf dry mass basis	$mg\ g_{DM}^{-1}$	3	3
C_{mass}	Leaf carbon content on leaf dry mass basis	$mg\ g_{DM}^{-1}$	3	3
$\Delta^{13}C$	Bulk leaf carbon isotope discrimination	‰	3	3

Italy) coupled with a continuous flow isotope ratio mass spectrometer (Delta S, Finnigan MAT, Bremen, Germany). Carbon isotope composition was expressed relative to the Vienna Pee Dee Belemnite (VPDB) standard as (Craig 1957)

$$\delta^{13}C = \left[\frac{R_{sample} - R_{standard}}{R_{standard}}\right] \times 1000\ (‰),$$

where R_{sample} and $R_{standard}$ refer to the $^{13}CO_2/^{12}CO_2$ ratios of the sample and the standard, respectively. The accuracy of $\delta^{13}C$ measurements was ±0.06‰.

$\Delta^{13}C$ between atmospheric CO_2 (δ_{air} assumed to be equal to −8‰) and plant material (δ_{plant}) was then calculated according to Farquhar and Richards (1984) as

$$\Delta^{13}C = \frac{\delta_{air} - \delta_{plant}}{[1 + (\delta_{plant}/1000)]}(‰)$$

Total carbon and nitrogen contents were determined from the same samples and were expressed on a dry mass basis (C_{mass} and N_{mass}, $mg\ g_{DM}^{-1}$).

Statistical analyses

Statistical analyses were performed using the R software (R Development Core Team 2012). Data required no transformation to meet the assumption of homoscedasticity and normal distribution of residuals. All tests were considered significant at $P < 0.05$. Individual data were analysed using the following linear models:

(i) for adjustment of individual data to block effects: $Y_{ijk} = \mu + B_i + G_{j(k)} + e_{ijk}$, where Y_{ijk} refers to individual performance, μ is the general mean, B_i is the effect of block i considered as fixed, $G_{j(k)}$ is the effect of the genotype j nested under metapopulation k considered as random and e_{ijk} is the residual error.

(ii) To characterize genetic variation expressed within each metapopulation in each common garden, a random model was used: $Y'_{ijk} = \mu + G_j + e'_{ijk}$ where Y'_{ijk} refers to the individual tree performance adjusted to block effects, G_j is the effect of the genotype j considered as random and e'_{ijk} is the residual error.

(iii) To evaluate metapopulation differentiation in each common garden, the following random model was used: $Y'_{ijk} = \mu + M_k + G_{j(k)} + e''_{ijk}$ where M_k is the effect of the metapopulation k considered as random, $G_{j(k)}$ is the effect of the genotype j nested under metapopulation k considered as random and e''_{ijk} is the residual error.

(iv) Prior to exploring $G \times E$ interactions, a Levene's test was performed and indicated that genetic and residual variances were not homoscedastic between metapopulations and common gardens. Therefore, $G \times E$ interactions were investigated for each metapopulation separately using the following

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random model: $Y'_{ijkl} = \mu + G_j + E_l + (G \times E)_{jl} + e_{ijkl}$ where G_j is the effect of the genotype j considered as random, E_l is the effect of the environment l considered as random, $(G \times E)_{jl}$ is the genotype by environmental interaction effect considered as random and e_{ijkl} is the residual error.

All the variance components from within and between common gardens models were estimated using the restricted maximum likelihood method. Broad-sense heritability was calculated on an individual basis (H^2_{ind}) for each metapopulation in each common garden according to the following equation: $H^2_{ind} = \sigma^2_G/(\sigma^2_G + \sigma^2_e)$ where σ^2_G and σ^2_e are genetic and residual variance components estimated for each metapopulation in each common garden. Standard errors associated with values of H^2_{ind} were calculated as described by Singh et al. (1993). The coefficient of genetic variation (CV_G) was calculated as the ratio of genetic standard deviation to the mean and was expressed as a percentage. Levels of population differentiation for quantitative traits are classically estimated through the coefficient of genetic differentiation (Q_{ST}), which is defined as the proportion of additive genetic variance attributable to differences between populations (Spitze 1993). Given that the present study was conducted with unrelated cloned individuals, we did not have access to additive genetic variation but to total genetic variation. Thus, the coefficient of metapopulation differentiation was estimated for each trait in each common garden as: $Q_{ST} = \sigma^2_M/(\sigma^2_M + 2 \times \sigma^2_G)$ with σ^2_G and σ^2_M representing the within- and among-metapopulations genetic variance components. The patterns of metapopulation differentiation were investigated by computing pairwise Q_{ST} across metapopulations.

When $G \times E$ interaction variance was significantly different from zero, it was further partitioned into two types of interactions according to method 1 developed by Muir et al. (1992): (i) interaction due to heterogeneous variances between common gardens and (ii) interaction due to changes in genotype ranking between common gardens. The stability of genotype and metapopulation ranking between the two common gardens was assessed by computing Spearman's rank coefficients (r_s) on a genotypic and metapopulation mean basis, respectively. Phenotypic relationships between leaf traits were analysed on a genotypic mean basis for each metapopulation in each common garden and described using linear regression analysis [Pearson's correlation coefficients (r_p)].

Results

Phenotypic variation in the two common gardens

All traits related to leaf dimensions (i.e., lamina length, lamina width, lamina perimeter, LA and petiole length) were highly correlated (data not shown). Data obtained for lamina length, lamina width, lamina perimeter and petiole length are not presented because they yielded comparable results to those obtained for LA. Most leaf traits differed between ORL and SAV (Figure 1). In SAV, leaves displayed a larger area than in ORL (102 ± 2 vs 34 ± 1 cm^2, respectively; Figure 1a). The amplitude of variation between extreme genotypic means for LA was greater in SAV than in ORL (228 vs 87 cm^2, respectively; Figure 1a). Lamina shape also differed between the two common gardens: leaves collected in SAV exhibited lower values of P : LA than in ORL (0.42 ± 0.01 vs 0.72 ± 0.01 cm^{-1}, respectively), indicating that leaves tended to have a more circular shape in SAV than in ORL (Figure 1b). Contrary to what was observed for LA, the range of variation between extreme genotypic means for lamina shape was lower in SAV than in ORL (0.41 vs 0.80 cm^{-1}, respectively; Figure 1b). Leaf structural and functional traits also differed between ORL and SAV: in the latter, leaves displayed higher SLA (126.6 ± 0.5 vs 90.4 ± 0.5 $cm^2\ g^{-1}$, respectively), higher nitrogen (32.7 ± 0.2 vs 14.6 ± 0.1 mg g^{-1}, respectively) and carbon content per mass unit (456.7 ± 0.4 vs 426.8 ± 0.6 mg g^{-1}) and lower $\Delta^{13}C$ values (19.09 ± 0.04 vs 21.55 ± 0.05‰) than in ORL (Figure 1c–f). The amplitude of variation between extreme genotypic means was similar between sites for $\Delta^{13}C$ (4.50‰; Figure 1f) while it was greater in SAV for SLA (56.1 vs 43.6 $cm^2\ g^{-1}$) and N_{mass} (20.4 vs 12.6 mg g^{-1}) and lower for C_{mass} (43.1 vs 69.2 mg g^{-1}) (Figure 1c–e).

Genetic variation within metapopulations, G × E interactions and relationships between traits

In each common garden and for each metapopulation, significant differences between genotypes were detected for all traits, except for N_{mass} and C_{mass} (Figure 1). When estimated on an individual basis, H^2_{ind} varied among traits but in a site-dependent manner (Figure 2a). At ORL, the highest H^2_{ind} values were recorded for $\Delta^{13}C$, while at SAV they were recorded for LA and lamina shape (P : LA) (Figure 2a). Irrespective of the common garden, N_{mass} and C_{mass} always displayed low values of H^2_{ind} (Figure 2a), explained by a low level of genetic variation and a higher influence of environmental variations. Standard errors associated with values of H^2_{ind} [$SE(H^2_{ind})$] never exceeded 0.13 ($0.05 \leq SE(H^2_{ind}) \leq 0.13$) regardless of the trait, the metapopulation or the common garden considered. On average, over all metapopulations and in comparison with ORL, heritability increased in SAV for the traits related to leaf size (i.e., LA) and leaf shape (i.e., P : LA), while it decreased for other structural and functional leaf traits (i.e., SLA and $\Delta^{13}C$) (Figure 2a). The CV_G was constant between ORL and SAV for most of the traits, showing that the amplitude of genetic variation was similar between the two common gardens (Figure 2b).

The relative contribution of $G \times E$ interactions to the total phenotypic variation was weak ($\sigma^2_{G\times E}/\sigma^2_P < 15\%$) for all metapopulations and traits (Table 3). For all the studied traits, the relative contribution of $G \times E$ effects to the total phenotypic variation reached the same level or was even greater than genetic effects for most of the metapopulations (Table 3). However, it is worth

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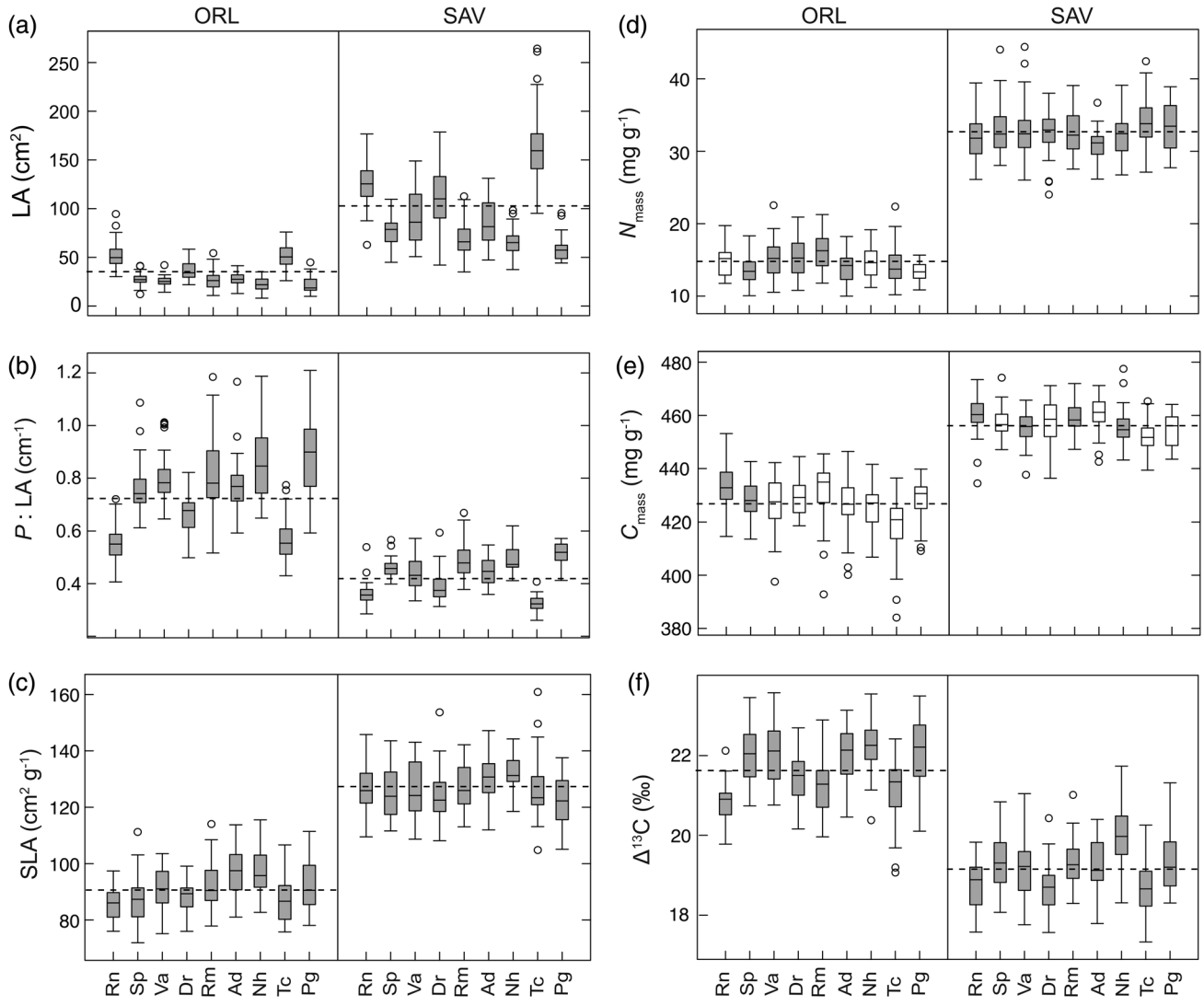

Figure 1. Phenotypic variation of leaf traits in the two common gardens: ORL and SAV. Box plots are shown for (a) LA, (b) the ratio between lamina perimeter and lamina area (P : LA), (c) specific leaf area (SLA), (d) leaf nitrogen content on leaf dry mass basis (N_{mass}), (e) leaf carbon content on leaf dry mass basis (C_{mass}) and (f) bulk leaf carbon isotope discrimination ($\Delta^{13}C$). Each box is based on block-adjusted genotypic means and represents the quartile below (Q_1) and above (Q_3) the median value. Vertical bars represent minimum and maximum values. Genotypes with values 1.5 times away from the top of the interquartile range (Q_3–Q_1) are represented by open circles. Boxes filled in grey indicate that the genetic variance within a metapopulation was different from zero. For all traits, the number of genotypes used to construct box plots for the two common gardens (ORL/SAV) is: Rn, $n = 31/32$; Sp, $n = 27/26$; Va, $n = 30/31$; Dr, $n = 30/30$; Rm, $n = 44/43$; Ad, $n = 30/31$; Nh, $n = 28/28$; Tc, $n = 62/62$; Pg, $n = 26/24$. The horizontal dashed lines represent the general mean for each trait in each common garden. Rn, Rhin; Sp, Saint-Pryvé; Va, ValAllier; Dr, Dranse; Rm, Ramières; Ad, Adour; Nh, Nohèdes; Tc, Ticino; Pg, Paglia.

noting that the ratio between $G \times E$ and genetic variances ($\sigma^2_{G\times E}/\sigma^2_G$) varied across metapopulations, but in a trait-dependent manner (Table 3). For LA and SLA and for some metapopulations, this ratio reached up to 7.1 and 11.5, respectively, while it averaged ~1 and did not exceed 1.5 for $\Delta^{13}C$ (Table 3, Figure 3). For LA, $G \times E$ interactions were explained by both changes in genotype ranking and increased variation in SAV (Figure 3a). For SLA and $\Delta^{13}C$, $G \times E$ interactions were mainly attributed to changes in genotype ranking between the two common gardens, which was further confirmed by the low-to-moderate values of Spearman's rank coefficient generally observed for these traits (Table 3, Figure 3b and c). For most of the metapopulations in the two common gardens, relationships between traits were weak and mostly non-significant such that no clear relationship could be evidenced (see Figure S2 available as Supplementary Data at *Tree Physiology* Online).

Genetic differentiation among metapopulations

Regardless of the common garden, metapopulations differed significantly for all leaf traits (Figure 1). The percentage of phenotypic variation explained by differences among metapopulations varied largely among traits: it accounted for 40–60% of the phenotypic variation for LA and P : LA while it was <30% for SLA and $\Delta^{13}C$ (Figure 4).

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The percentage of phenotypic variance explained by differences among metapopulations appeared to be modulated by the environment in a trait-dependent manner: it was greater in SAV for LA (59 vs 44%) and P : LA (52 vs 40%) while the opposite trend was observed for SLA (3 vs 17%) and $\Delta^{13}C$ (17 vs 29%) (Figure 4). Graphical inspection, combined with the estimation of Spearman's rank coefficients among metapopulation means, indicated that the metapopulation ranking was highly conserved between the two common gardens for the traits related to leaf size (i.e., LA) and leaf shape (i.e., P : LA), while it was modified between ORL and SAV for structural and functional leaf traits (i.e., SLA and $\Delta^{13}C$) (see Figure S3 available as Supplementary Data at *Tree Physiology* Online). For $\Delta^{13}C$, it is worth noting that the relationship among metapopulation means observed in ORL and SAV tended to be triangular: metapopulations with low values of $\Delta^{13}C$ in ORL also showed low values for this trait in SAV whereas metapopulations with high values of $\Delta^{13}C$ in ORL exhibited a great range of variation for this trait in SAV (see Figure S3 available as Supplementary Data at *Tree Physiology* Online).

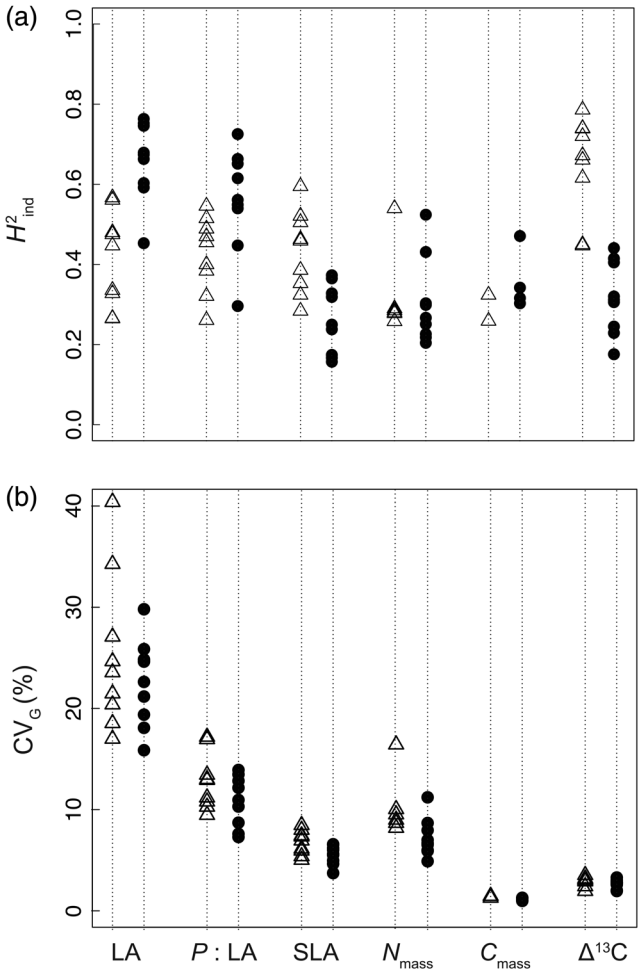

Figure 2. Broad-sense heritability estimated on an individual basis (H^2_{ind}) (a) and CV_G (b) calculated for each trait for each metapopulation in each common garden, ORL (open triangles) and SAV (filled circles). For both common gardens, values of H^2_{ind} and CV_G were calculated when genetic variance within a metapopulation was different from zero. Standard errors associated with values of H^2_{ind} were not plotted for the clarity of the figure. See Table 2 for trait abbreviations.

The level of metapopulation differentiation (Q_{ST}) for a given trait was comparable between ORL and SAV, but it largely varied among traits (Figure 4). The highest Q_{ST} values were detected for LA ($Q_{ST} = 0.50$ at ORL and 0.51 at SAV) and P : LA ($Q_{ST} = 0.43$ at ORL and 0.48 at SAV) and the lowest one for SLA ($Q_{ST} = 0.19$ at ORL and 0.05 at SAV) (Figure 4). For two of the most differentiated traits, LA and $\Delta^{13}C$, the differences between metapopulations could not be described according to a latitudinal cline irrespective of the common garden (Figure 1a and f). Therefore, the patterns of pairwise metapopulation differentiation were investigated to evaluate how metapopulations clustered for these traits (Figure 5). The geographical pattern of metapopulation differentiation was highly conserved between the two common gardens for LA (Figure 5a). The metapopulations clustered into three major groups: the Eastern metapopulations, Rhin, Dranse and Ticino exhibited the highest values for LA, while the lowest ones were obtained for the metapopulations Nohèdes, Ramières and Paglia; intermediate values of LA were observed for the metapopulations Adour, Saint-Pryvé and ValAllier (Figures 1a and 5a). Although the pattern of metapopulation differentiation for $\Delta^{13}C$ differed between the two common gardens, the metapopulations Rhin, Dranse and Ticino always displayed the lowest values for $\Delta^{13}C$, while the opposite trend was observed for the metapopulations Adour, Paglia, Saint-Pryvé and ValAllier (Figures 1f and 5b).

Table 3. Phenotypic variance components and stability of genotype ranking between the two experimental sites for leaf traits. $\sigma^2_P, \sigma^2_E, \sigma^2_G, \sigma^2_{G\times E}$, phenotypic, environmental, genetic and genotype by environmental interaction variance components; $N_{Metapop.}$, number of metapopulations for which variance components or Spearman's rank coefficients (r_s) were significantly different from zero; median, minimum and maximum calculated on the $N_{Metapop.}$ estimates; see Table 2 for trait abbreviations.

Trait	σ^2_E/σ^2_P (%)			σ^2_G/σ^2_P (%)			$\sigma^2_{G\times E}/\sigma^2_P$ (%)			$\sigma^2_{G\times E}/\sigma^2_G$			r_s		
	$N_{Metapop.}$	Median	Min.–max.	$N_{Metapop.}$	Median	Min.–max.	$N_{Metapop.}$	Median	Min.–max.	$N_{Metapop.}$	Median	Min.–max.	$N_{Metapop.}$	Median	Min.–max.
LA	9	81.4	75.5–87.5	8	4.2	1.2–10.6	9	6.4	2.9–13.4	8	1.4	0.3–11.5	6	0.54	0.46–0.79
P : LA	9	66.8	37.3–78.3	8	6.0	0.9–10.2	6	7.2	5.6–9.2	6	1.8	0.7–6.0	6	0.50	0.41–0.74
SLA	9	82.4	74.6–86.7	9	2.7	0.2–3.2	5	3.5	1.9–5.1	5	1.5	0.6–7.1	3	0.36	0.30–0.60
$\Delta^{13}C$	9	80.1	74.8–85.1	9	5.1	2.8–9.2	6	4.2	3.9–6.0	6	1.1	0.5–1.5	7	0.44	0.28–0.61

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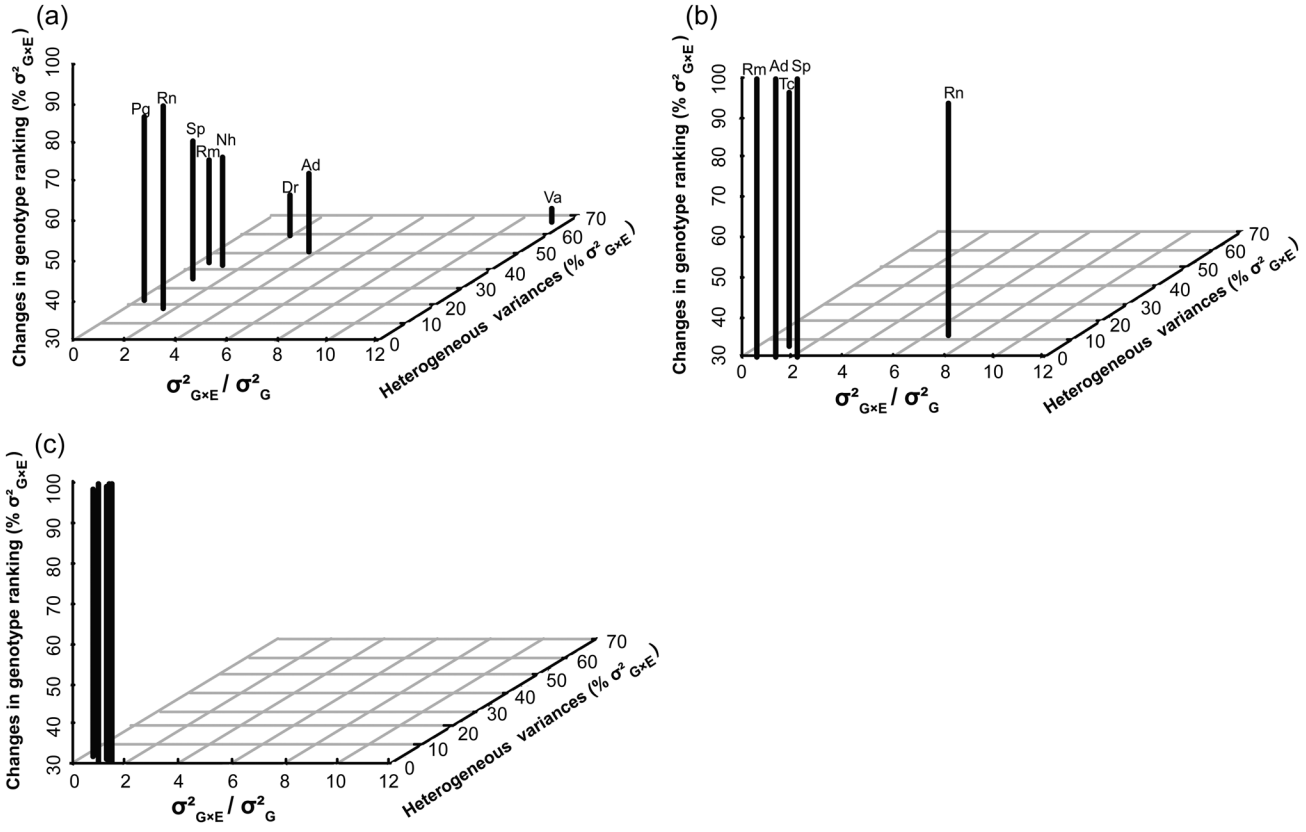

Figure 3. Importance of genotype by environment ($G \times E$) interactions relative to genetic effects ($\sigma^2_{G\times E}/\sigma^2_G$) and partitioning of $G \times E$ interactions into the part due to heterogeneous variances and the part due to changes in genotype ranking between the two common gardens, ORL and SAV. Results are shown for (a) LA, (b) specific leaf area and (c) bulk leaf carbon isotope discrimination. Results are illustrated for the metapopulations for which $G \times E$ interaction and genetic variance components were significantly different from zero. For bulk leaf carbon isotope discrimination, metapopulations are not labelled because all metapopulations are confounded. Metapopulation abbreviations are as in Figure 1.

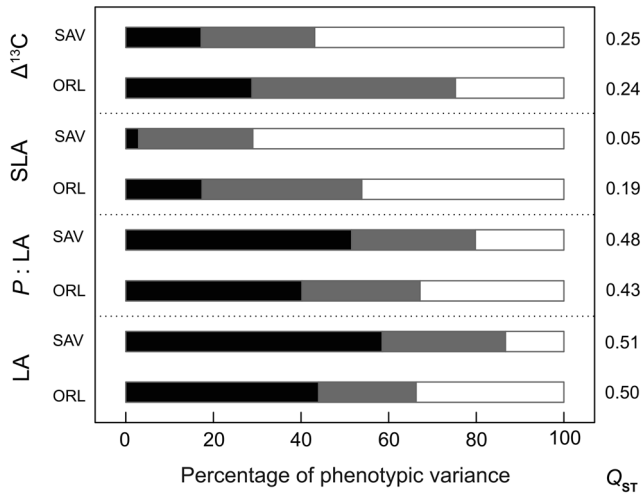

Figure 4. Partitioning of phenotypic variance for leaf traits in the two common gardens, ORL and SAV. The bars represent phenotypic variance components expressed as percentage: genetic variance (tinted bars) and residual variance (open bars). The genetic variance component was further partitioned into metapopulation (black bars) and genotype within metapopulation (grey bars) components. The coefficients of metapopulation differentiation (Q_{ST}) are given. See Table 2 for trait abbreviations.

Discussion

In this paper, we evaluated the relative contribution of genetic and environmental effects to the phenotypic variation expressed for leaf morphology, leaf structure and $\Delta^{13}C$ in European *P. nigra* metapopulations. The study was conducted in two common gardens, one located in Central France (ORL) and the other one in Northern Italy (SAV), such that robustness of metapopulation differentiation over environments could be tested. In SAV, the leaves exhibited, on average, a higher individual area, a more circular shape, a higher SLA and a lower value of $\Delta^{13}C$. These variations were attributed to the confounded effect of a higher soil fertility (Liu and Dickmann 1992, Ibrahim et al. 1997) and an invigorating effect of shoot pruning on SAV (Tschaplinski and Blake 1989, 1995, Dickmann et al. 1996).

Partitioning of phenotypic variation within metapopulations

Our sampling strategy enabled us to estimate genetic parameters for each metapopulation in each common garden, which is consistent with the fact that genetic variance components and

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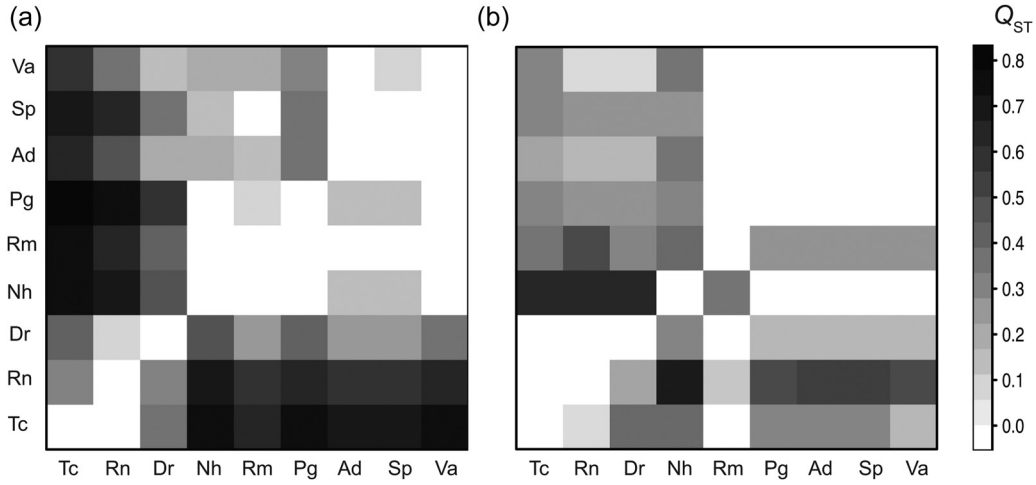

Figure 5. Patterns of pairwise genetic differentiation of metapopulations estimated with phenotypic data of two quantitative traits: (a) LA and (b) bulk leaf carbon isotope discrimination. Metapopulations were plotted on both *X*-axis and *Y*-axis and were ordered from the left to the right (*X*-axis) and from the bottom to the top (*Y*-axis) according to their level of differentiation with all the other metapopulations. Pairwise genetic differentiation of metapopulations was estimated for the two common gardens, ORL (below the diagonal) and SAV (above the diagonal). Colouration is indicative of the coefficients of genetic differentiation according to the bar on the right. Metapopulation abbreviations are as in Figure 1.

heritability of traits are defined relative to one given population in a given environment (Visscher et al. 2008). The moderate-to-high values of H^2_{ind} reported for most leaf traits ($0.18 \leq H^2_{ind} \leq 0.79$) attested that the micro-environmental influences on phenotypic expression have been well-controlled in the two common gardens to allow the detection of significant genetic variation within the nine *P. nigra* metapopulations.

For most of the studied metapopulations, the phenotypic variance for leaf traits was significantly modulated by environmental conditions, as attested not only by environmental effects but also by a $G \times E$ interaction variance comparable to or even greater than total genetic variance. However, the partitioning of the $G \times E$ interaction variance revealed contrasted interaction patterns depending on leaf traits. For LA, these interactions were explained by both changes in genotype ranking between the two common gardens and increased variation in SAV, while they were essentially explained by changes in genotype ranking between common gardens for SLA and $\Delta^{13}C$. These results indicate that the nine *P. nigra* metapopulations presented a substantial individual variation in the pattern of plastic responses for leaf traits to environmental variations.

As a pioneer tree species, the successful establishment of black poplar in the riparian habitat partly depends on its ability to rapidly colonize its environment, which may notably depend on the variation expressed for phenotypic traits involved in resource acquisition and plant functioning, such as LA. Thus, the maintenance in *P. nigra* metapopulations of a high level of phenotypic plasticity for LA may contribute to high whole-plant plasticity and thus optimal population response to varying environmental conditions. The huge increase of variation observed for LA in SAV indicates that the contrast between *P. nigra* genotypes for this trait was exacerbated and may reflect different strategies in crown and leaf area development. Crown architecture determines leaf display, leaf distribution and canopy density and is therefore a critical determinant of whole-plant light interception (Hallé et al. 1978). A major part of the canopy architecture is influenced by the branching pattern. Poplars produce both proleptic and sylleptic branches. Proleptic branches are produced from buds that experienced a period of winter dormancy, while sylleptics develop from meristems during the same growing season in which they were formed (Remphrey and Powell 1985). Sylleptic branches are characterized by a larger and faster development of leaf area when compared with the main stem axis (Scarascia-Mugnozza et al. 1989, Ceulemans et al. 1990, Zeleznik 2007). In addition, the large leaf area carried by sylleptic branches exports more carbon to the main stem and roots than main stem leaves, which much contribute to height growth (Scarascia-Mugnozza et al. 1999). Leaf area distribution by main stem axis vs sylleptic branches varies substantially among poplar genotypes (Ceulemans et al. 1990, Dunlap et al. 1995, Gielen et al. 2002, Zeleznik 2007, Broeckx et al. 2012) as well as in response to environmental conditions (Wu and Stettler 1998, Marron et al. 2006). These variations are known to play a key role in determining growth performances, a large number of sylleptics being generally associated with high growth performances (Ceulemans et al. 1990, Rae et al. 2004, Marron et al. 2006). Future research in black poplar should therefore adopt a better integrated approach by combining measurements at the leaf level but also at the whole-plant level to gain insights into the phenotypic plasticity of leaf size as well as of plant branchiness and crown architecture in response to environmental variations.

$\Delta^{13}C$ is a complex and composite trait reflecting primarily the balance between photosynthetic capacities and stomatal

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conductance to water vapour (in poplars, for example, Monclus et al. 2006, Fichot et al. 2011, Rasheed et al. 2013). Plastic responses for $\Delta^{13}C$ are therefore potentially governed by the underlying responses of other morphological, structural and physiological components, including leaf nitrogen content and allocation to the photosynthetic apparatus, leaf density and/or leaf thickness, mesophyll conductance, stomatal density and stomatal morphology. The contribution of these components to the plastic responses for $\Delta^{13}C$ is likely to vary depending on environment and genetic background, which may favour the detection of significant $G \times E$ interactions. The examination of $G \times E$ interactions for $\Delta^{13}C$ in forest tree species, including poplars, brought contrasting results, with some studies reporting significant $G \times E$ interactions (Cregg et al. 2000, Ponton et al. 2002 in response to varying irradiance levels, Corcuera et al. 2010, Chamaillard et al. 2011 in response to site effect, Dillen et al. 2011, Toillon et al. 2013) while others failed to evidence such interactions (Johnsen et al. 1999, Ponton et al. 2002 in response to varying water availability, Chamaillard et al. 2011 in response to varying water availability). As suggested by Dillen et al. (2011), the contrasting results for $G \times E$ interactions for $\Delta^{13}C$ could be partly explained by differences in (i) plant material, genetic background and relatedness between studied genotypes, (ii) the number of genotypes studied and (iii) the nature of environmental variables driving plastic responses for $\Delta^{13}C$.

Genetic differentiation among metapopulations

Genetic differentiation among the nine *P. nigra* metapopulations was evidenced in the two common gardens for most of the studied leaf traits despite the substantial level of genetic variation expressed within metapopulations. In forest tree species, many studies examining jointly the levels and the patterns of population differentiation for quantitative traits have been conducted under single environments. Our two-site common garden experiment therefore enabled us to assess whether the patterns of metapopulation differentiation were conserved in different environments.

The nine *P. nigra* metapopulations were highly differentiated for LA in the two common gardens ($Q_{ST} = 0.50$ at ORL and 0.51 at SAV). This level of genetic differentiation was of the same order of magnitude as the one reported in a set of 14 *P. nigra* populations, covering a comparable geographic range, for growth cessation ($Q_{ST} = 0.59$) and bud set ($Q_{ST} = 0.60$) (Rohde et al. 2011), two traits known to play a key role for local adaptation of natural populations of forest tree species (Howe et al. 2003, Alberto et al. 2013) including poplars (Hall et al. 2007, Keller et al. 2011, Evans et al. 2014). Interestingly, the pattern of metapopulation differentiation for LA was highly conserved between the two common gardens, suggesting that the evolutionary forces that have shaped this pattern have left a strong imprint, such that the differences between metapopulations were robust to environmental variations. $Q_{ST} - F_{ST}$ comparisons, i.e., comparative studies of the divergence of populations for quantitative traits and the corresponding differentiation based on neutral molecular markers, provide a means for researchers to infer the action of natural selection on phenotypic traits (Merilä and Crnokrak 2001). Microsatellites genotyping of a set of European *P. nigra* populations partially overlapping with the set studied here has revealed that European populations of black poplar are weakly differentiated, with pairwise F_{ST} values ranging from 0.002 to 0.158 among populations (Smulders et al. 2008). This range of pairwise F_{ST} values was much lower than the range of pairwise Q_{ST} observed for LA in the two common gardens (58% of pairwise Q_{ST} values ranging from 0.20 to 0.76 at ORL and 54% of pairwise Q_{ST} values ranging from 0.21 to 0.78 at SAV), which led us to hypothesize that the pattern of metapopulation differentiation for this trait could have been partly shaped by directional selection. In *Q. suber*, Ramírez-Valiente et al. (2009) showed that leaf size was highly differentiated among 13 natural populations ($Q_{ST} = 0.46$) and the Q_{ST} value for this trait was much higher than the F_{ST} estimate (0.032), providing evidence of adaptive differentiation. Comparable results were obtained for petiole length in natural populations of *P. balsamifera* ($Q_{ST} = 0.70$; Keller et al. 2011). On the basis of chloroplast DNA structuration patterns, Cottrell et al. (2005) have suggested that black poplar had several glacial refugia in Spain, in Italy and/or in the Balkans from which Southern France and the Eastern part of Europe were, respectively, colonized after the ice age. The fact that the three Eastern *P. nigra* metapopulations (i.e., Rhin, Dranse and Ticino) clustered together for LA suggests that post-glacial events and subsequent routes of recolonization may have also played a role in shaping the geographical pattern of genetic variation for LA in black poplar.

Natural populations of different forest tree species, including poplar species, have been found to exhibit low-to-moderate levels of genetic differentiation for $\Delta^{13}C$ ($0.06 \leq Q_{ST} \leq 0.21$) (Ramírez-Valiente et al. 2009, Keller et al. 2011, Lamy et al. 2011). Our results further confirmed this trend since the nine *P. nigra* metapopulations were moderately differentiated for $\Delta^{13}C$ in the two common gardens ($Q_{ST} = 0.24$ at ORL and 0.25 at SAV). We also evidenced that the pattern of metapopulation differentiation for $\Delta^{13}C$ was not robust to environmental variations between the two common gardens. Given that $\Delta^{13}C$ is a composite trait, the physiological components driving the variations observed for $\Delta^{13}C$ may vary depending on environmental conditions, which may be responsible for significant changes of population clustering across environments. In forest tree species, the variations observed for $\Delta^{13}C$ have been found to be under the control of several quantitative trait loci (QTLs; 4–10 QTLs) with small-to-moderate effects (2–30% of the total phenotypic variation) and not always stable over time and environmental conditions (Brendel et al. 2002, Casasoli et al. 2004, Rönnberg-Wästljung et al. 2005, Rae et al. 2009, Monclus et al.

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2012). The complex genetic architecture underlying the variations of $\Delta^{13}C$ may also explain the difficulty of observing stable pattern of population differentiation for this trait across environments. As suggested by Muir et al. (2014), phenotypic traits that are highly polygenic, such as $\Delta^{13}C$, may respond to selection less readily than traits governed by few loci with large effects because of the potential existence of significant pleiotropy and correlations with other traits, and because large genetic changes induced by directional selection are necessary to achieve phenotypic changes.

Conclusions

Our two-site common garden experiment revealed that the nine *P. nigra* metapopulations present substantial genetic variation and phenotypic plasticity for leaf morphology, leaf structure and $\Delta^{13}C$, which both represent potentially significant determinants of the capacities of populations to respond, on a short-term basis and over generations, to environmental variations. The patterns of metapopulation differentiation observed for LA and $\Delta^{13}C$ differed from the latitudinal cline previously reported for bud set in a set of comparable natural populations of black poplar, suggesting that the patterns of genetic variation for these traits are shaped by different evolutionary and/or environmental drivers. Finally, our findings also evidenced that the three Eastern metapopulations, Rhin, Dranse and Ticino, were highly differentiated from all the other metapopulations for both LA and $\Delta^{13}C$, exhibiting on average the largest LA and the lowest $\Delta^{13}C$. This specific multivariate behaviour could be the product of co-selection on a set of traits that maximize plant fitness under specific environmental conditions. These results also suggest that glacial refugia and subsequent routes of recolonization also played a role in shaping the patterns of genetic variation for leaf traits in *P. nigra* metapopulations. Recent advances in next-generation DNA sequencing technologies have made possible the development of high-throughput single nucleotide polymorphism (SNP) genotyping platforms that allow for simultaneous interrogation of thousands of SNPs in poplar (Geraldes et al. 2013). Such resources will provide a valuable tool to develop association genetics studies and to identify potential signatures of selection in candidate genomic regions for LA and $\Delta^{13}C$ in black poplar.

Supplementary data

Supplementary data for this article are available at *Tree Physiology* Online.

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Conflict of interest

None declared.

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