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Diversity of water use efficiency among *Quercus robur* genotypes: contribution of related leaf traits

Magali ROUSSEL, Didier LE THIEC, Pierre MONTPIED, Nathalie NINGRE, Jean-Marc GUEHL, Oliver BRENDEL*

INRA, UMR1137 INRA - Nancy-Université "Écologie et Écophysiologie Forestières", IFR 110 54280 Champenoux, France

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Abstract

• Previously, a large intra-specific diversity and a tight genetic control have been shown for $\Delta^{13}\text{C}$ (carbon isotope discrimination) in a pedunculate oak (*Quercus robur* L.) family, which is an estimator for intrinsic water use efficiency (W_i), a complex trait defined as the ratio of net CO_2 assimilation rate (A) to stomatal conductance for water vapour (g_s).

• In the present study, twelve genotypes with extreme phenotypic values of $\Delta^{13}\text{C}$ were selected within this family to (i) assess the stability of genotype differences across contrasting environments and for different measures W_i ; (ii) quantify the relationship between $\Delta^{13}\text{C}$ and W_i within this family; (iii) identify which leaf traits drive the diversity in W_i observed in this family.

• Genetic variability of $\Delta^{13}\text{C}$ and W_i was largely independent from different temporal integration scales and their correlation was found to be strong ($R^2 = 88\%$ for leaf sugars) within this family.

• Weak correlations between measures of W_i with estimators of photosynthetic capacity, suggest a minor role of the latter in the diversity of W_i .

• However, the tight correlation between g_s and $\Delta^{13}\text{C}$ as well as W_i , and the related genotypic variation in stomatal density, suggest that the genotypic diversity in W_i within this pedunculate oak family might be due to differences in g_s .

Résumé – Diversité de l'efficacité d'utilisation de l'eau entre différents génotypes de *Quercus robur* : contributions des traits foliaires.

• Une large diversité intra-spécifique et un fort contrôle génétique ont été mis en évidence pour $\Delta^{13}\text{C}$ (discrimination isotopique du carbone) dans une famille de chêne pédonculé (*Quercus robur* L.). $\Delta^{13}\text{C}$ est un estimateur de l'efficacité intrinsèque d'utilisation de l'eau (W_i), un caractère complexe défini comme le rapport entre l'assimilation nette de CO_2 (A) et la conductance stomatique pour la vapeur d'eau (g_s).

• Douze génotypes présentant des valeurs phénotypiques extrêmes de $\Delta^{13}\text{C}$ ont été sélectionnés dans cette famille pour (i) évaluer la stabilité des différences génotypiques dans des environnements contrastés et pour différents estimateurs de l'efficacité d'utilisation de l'eau ; (ii) quantifier la relation entre $\Delta^{13}\text{C}$ et W_i dans cette famille ; (iii) identifier quels caractères foliaires sont impliqués dans la diversité de W_i observée dans cette famille.

• La variabilité génétique de $\Delta^{13}\text{C}$ et W_i était largement indépendante des différentes échelles d'intégration temporelles et leur corrélation était forte dans cette famille ($R^2 = 88\%$ lorsque $\Delta^{13}\text{C}$ était mesuré dans les sucres foliaires).

• Les faibles corrélations entre W_i (ou $\Delta^{13}\text{C}$) et des estimateurs de la capacité photosynthétique suggèrent un rôle mineur de celle-ci dans la diversité de W_i .

• Par contre, la corrélation étroite entre g_s et $\Delta^{13}\text{C}$ ainsi que W_i et la variation génétique de la densité stomatique, suggèrent que la diversité génétique de W_i dans cette famille de chêne pédonculé est liée à des différences de conductance stomatique g_s .

* Corresponding author: brendel@nancy.inra.fr

Abbreviations: $\Delta^{13}\text{C}$: ^{13}C isotope discrimination (‰); $\Delta^{13}\text{C}_{\text{su}}$: $\Delta^{13}\text{C}$ of extracted leaf sugars; $\Delta^{13}\text{C}_{\text{st}}$: $\Delta^{13}\text{C}$ of extracted leaf starch; $\Delta^{13}\text{C}_{\text{b}}$: $\Delta^{13}\text{C}$ of bulk leaf material; $\delta^{13}\text{C}$: carbon isotope composition (‰); $\delta^{13}\text{C}_{\text{air}}$: $\delta^{13}\text{C}$ of atmospheric CO_2 in the greenhouse; c_i : CO_2 mole fraction in intercellular air spaces ($\mu\text{mol mol}^{-1}$); c_a : atmospheric CO_2 mole fraction ($\mu\text{mol mol}^{-1}$); W_i : intrinsic water use efficiency ($\mu\text{mol mol}^{-1}$); A : net CO_2 assimilation rate ($\mu\text{mol m}^{-2} \text{s}^{-1}$); A_{sat} : light saturated A at ambient CO_2 concentration; g_s : stomatal conductance for water vapour ($\text{mol m}^{-2} \text{s}^{-1}$); V_{cmax} : apparent maximum carboxylation rate of Rubisco ($\mu\text{mol m}^{-2} \text{s}^{-1}$); N_{mass} : nitrogen content on a mass basis (mg g^{-1}); N_{area} : nitrogen content on an area basis (g m^{-2}); Chl : chlorophyll content (g m^{-2}); SD : stomatal density (mm^{-2}); SL : stomatal length (μm); SA : stomatal area (μm^2); SAI : stomatal area index (mm^{-1}); LMA : leaf mass-to-area ratio (g m^{-2}); TLT : total leaf thickness (μm); UET : upper epidermis thickness (μm); LET : lower epidermis thickness (μm); PMT : palisade mesophyll thickness (μm); SMT : spongy mesophyll thickness (μm).

1. INTRODUCTION

A large intra-specific variability of ^{13}C isotope discrimination ($\Delta^{13}\text{C}$) has been observed within natural stands of pedunculate oak (*Quercus robur* L.; Ponton et al., 2001), a widespread broad-leaved forest tree species from Western Europe. Leaf $\Delta^{13}\text{C}$ is a widely used estimator of intrinsic water use efficiency, W_i , defined as the ratio of net CO_2 assimilation rate (A) to stomatal conductance for water vapour (g_s). The predicted relationship between $\Delta^{13}\text{C}$ and W_i is negative (Farquhar and Richards, 1984) which has been observed for pedunculate and sessile oaks under different levels of shading (Ponton et al., 2002). W_i is a complex trait related to many physiological and structural leaf traits that directly or indirectly influence A or g_s . Genetic variation in W_i or $\Delta^{13}\text{C}$ has been shown within many tree species and was ascribed to variations in A (Johnsen and Major, 1995; Major and Johnsen, 1996; Xu et al., 2000), in g_s (Cregg et al., 2000) or in both (Farquhar et al., 1989).

Brendel et al. (2008) investigated the genetic determinism of water use efficiency in a pedunculate oak full-sib family comprising 278 siblings. They found in this progeny a 40% variation of W_i as estimated by leaf $\Delta^{13}\text{C}$. The genetic control of $\Delta^{13}\text{C}$ was oligogenic, with only a few Quantitative Trait Loci (QTL) explaining a large fraction of the observed variability of $\Delta^{13}\text{C}$ (30% to 50%). The detected QTL pointed to two major regions on the genetic map of this full-sib family. The first region explained 21–31% of the observed variability, and was detected with independent measurements conducted for three successive years, suggesting that only low QTL \times environment interactions occurred. The second region suggested an influence of photosynthetic capacity on the observed diversity in $\Delta^{13}\text{C}$ and explained 9% of the observed variability.

Variation in A , when independent from g_s , is due to variation in photosynthetic capacity. Theoretical considerations show that compounds involved in photosynthesis may account for well over three-quarter of total leaf nitrogen (Field and Mooney, 1986). Thus for different species photosynthetic capacity was found to correlate with total leaf nitrogen (Evans, 1989), including several oak species (Takashima et al., 2004). Leaf anatomy traits such as leaf density and thickness may also influence photosynthetic capacity (Niinemets, 1999) and modulate the photosynthesis-nitrogen relationships (Reich et al., 1998).

Physiological processes leading to stomatal aperture or closure are well known and recent progress has been made on genes involved in ABA and Ca^+ signalling (Webb and Baker, 2002) and stomatal development (Bergmann, 2006). However, the main causes driving diversity in g_s in natural populations are not yet well understood, both within and among species. Franks and Farquhar (2007) found an influence of the stomatal morphology of different species on maximum stomatal apertures and rapidity of movements. It has also been shown for temperate deciduous trees that the size of guard cells has an influence stomatal aperture and therefore contributes to the diversity of g_s (Aasamaa et al., 2001). Further it has been suggested that maximum stomatal conductance is influenced by stomatal density (Nobel, 1999). Little is however known on the causes of within species diversity of stomatal conductance.

To study genetic, intraspecific diversity, and to assess genetic determinism and control of traits, a large number of siblings and vegetative copies (several hundred individuals) are required. This puts a severe practical constraint on the number of leaf traits that can be measured. Therefore we focused on a smaller number of individuals while maximising the differences in W_i and $\Delta^{13}\text{C}$. To this end, a few individuals (called genotypes hereafter) were selected for their extreme phenotypic values as observed by Brendel et al. (2008), to cover the diversity detected within the pedunculate oak full-sib family. Two strategies were used to sample genotypes: first, using $\Delta^{13}\text{C}$ data measured during three successive years and second, using a combination of $\Delta^{13}\text{C}$ with leaf nitrogen and chlorophyll content data. With the first sampling strategy genotypes were selected that have accumulated alleles with positive (or negative) effects on $\Delta^{13}\text{C}$, independently from the physiological process affecting discrimination. The second strategy aimed at maximizing the diversity of $\Delta^{13}\text{C}$ caused by photosynthetic capacity, by selecting genotypes with simultaneously extreme phenotypic values for $\Delta^{13}\text{C}$, leaf nitrogen and chlorophyll content.

Although bulk leaf matter is often used for rapid estimates of $\Delta^{13}\text{C}$ (e.g. for QTL studies as in Brendel et al., 2008), it is an aggregate of different carbon containing compounds, each with different integration patterns of W_i . This can cause discrepancies between W_i estimated from leaf bulk $\Delta^{13}\text{C}$ and W_i estimated directly and instantaneously from gas exchange measurements. Soluble sugars and starch provide a $\Delta^{13}\text{C}$ signal with a short time-integration due to their high turn-over rate (few days) (Brugnoli et al., 1988). $\Delta^{13}\text{C}$ was therefore

assessed from three different pools of organic matter (bulk leaf matter, starch, soluble sugars) to downscale from field measurements (Brendel et al., 2008) to instantaneous gas exchange. Further, genetic variation of certain leaf traits could disturb the relationship between $\Delta^{13}\text{C}$ and intrinsic water use efficiency (W_i) by affecting the parameters of the theoretical model.

Therefore our main objectives were to:

- (i) assess the stability of genotype differences across contrasting environments and for different measures of intrinsic water use efficiency (W_i);
- (ii) quantify how genetic variation in $\Delta^{13}\text{C}$ relates to genetic variation in W_i within this family;
- (iii) identify which leaf traits drive the diversity in W_i observed in this family.

2. MATERIAL AND METHODS

2.1. Plant material and growth conditions

Genotypes used in this study were selected within a pedunculate oak progeny (F1 of 278 clones) which has been used several times for QTL detection (Brendel et al., 2008; Parelle et al., 2007; Scotti-Saintagne et al., 2004; 2005). Selection criteria were based on phenotypic data recorded under natural conditions at Bourran (South Western France) (Brendel et al., 2008). The traits used were carbon isotope discrimination of bulk leaf organic matter ($\Delta^{13}\text{C}_b$), chlorophyll (Chl) and nitrogen content (N_{area} , N_{mass}) of leaves. Genotypes with extreme phenotypes for $\Delta^{13}\text{C}$ (i.e. “low Δ ” or “high Δ ”) were selected on the basis of extreme values of leaf $\Delta^{13}\text{C}$ recorded during three years of measurements (2000 to 2002). Genotypes were chosen which showed extreme low or extreme high leaf $\delta^{13}\text{C}$ data for all three years. In parallel, genotypes with extreme phenotypes concurrently for $\Delta^{13}\text{C}$, chlorophyll concentration and leaf N (i.e. “low N” or “high N”) were selected on the basis of displaying simultaneously high values of leaf $\Delta^{13}\text{C}$ and low values of chlorophyll concentration and leaf nitrogen content recorded in 2001 and inversely. This selection was founded on the hypothesis that genotypes with low photosynthetic capacity, estimated by low values of leaf nitrogen and chlorophyll would show low W_i , and thus high leaf $\delta^{13}\text{C}$. Three genotypes were chosen for each of the extreme phenotypes: “low Δ ” (siblings 159, 171, 287), “high Δ ” (136, 170, 263), “low N” (151, 237, 351) and “high N” (259, 261, 267), forming a set of twelve genotypes overall.

Clonal copies of the selected genotypes were produced in summer 2003 by UMR BioGECO, INRA Pierroton (South Western France) and then transferred to a greenhouse at Champenoux near Nancy (North Eastern France) during December 2003. The experiment took place during summer 2004. The trees were transplanted to 10 L containers with a peat and sand mixture (1/1, v/v). A complete fertilisation (4.5 g L^{-1} of slow-release fertiliser Nutricote T100: N, P, K, Mg, 13,13,13,2 + trace elements and 0.2 g L^{-1} of lime) was provided. All individuals were watered to field capacity three times daily with deionised water. Air temperature control of the greenhouse was set to a minimum of $15 \text{ }^\circ\text{C}$ and a maximum of $25 \text{ }^\circ\text{C}$, otherwise within this range greenhouse temperature was following outside temperature

variations. Relative humidity ($68 \pm 12\%$, average \pm standard deviation), CO_2 concentration ($394 \pm 12 \mu\text{mol mol}^{-1}$) and daily global irradiance were continuously recorded in the greenhouse. The plants were grown under natural photoperiod. As pedunculate oaks show a periodical growth during the growing season (“flushes”), all measurements were done on unshaded adult second flush leaves.

Four vegetative copies (rooted cuttings) of each genotype were used as replicates. The complete experiment consisted of 40 plants overall that were placed in a greenhouse with a randomized complete block design. One vegetative copy of each genotype was placed in each block, with four blocks overall. Due to some mortality of rooted cuttings, especially in the phenotype “high Δ ”, the number of clonal copies of three genotypes was only one or two.

2.2. Extraction of soluble sugars and starch from leaves, and carbon isotope analysis

At the beginning of July, one leaf was harvested on each individual plant, oven-dried ($60 \text{ }^\circ\text{C}$) and ball-milled for carbon isotope analysis of bulk leaf matter. Additionally, three to five leaves were harvested separately for extraction of soluble sugars and starch, freeze-dried for 24 h and then ball-milled. The method of extraction was adapted from Dickson and Larson (1975). Extraction was carried out using 200 mg of leaf powder with 2 mL of a mixture of methanol/chloroform/water (12:5:3, v/v/v). After shaking with a vortex mixer, and 30 min at ambient temperature, tubes were centrifuged for 10 min at 2000 g and $4 \text{ }^\circ\text{C}$. The supernatant was recovered, a second extraction was run on the pellet and the second supernatant was added to the first. Then 1 mL of methanol 60% was added to the pellet, and this solution was stored at $-20 \text{ }^\circ\text{C}$ for starch extraction. Sugar extraction continued using the supernatant by addition of 2 mL of a mixture methanol/chloroform (1:1 v/v) and 1.2 mL of distilled water. After vortexing, and centrifugation (5 min, 2000 g, $4 \text{ }^\circ\text{C}$), the upper phase was collected and 0.5 mL of methanol (60%) was added to the remainder. After vortexing again, and centrifugation, the supernatant was retained and added to the first one. Methanol and water were then evaporated overnight in a vacuum concentration system (1 mbar, $30 \text{ }^\circ\text{C}$, Heto, Denmark). The pellet was rehydrated with 0.5 mL distilled water and vortexed. Soluble sugars were then separated from the aqueous extract through a multilayer extraction column, consisting of (i) PVPP (Polyvinylpyrrolidone, Sigma-Aldrich, Germany) to retain phenols and tannins; (ii) Dowex -50 (H^+) resin (Sigma-Aldrich, Germany) used for separation of amino acids from organic acids and sugars and (iii) Amberlite IRA-402 (Cl^-) resin (Sigma-Aldrich, Germany) used for separation of organic acids from sugars. The soluble sugars were eluted with 8 mL of distilled water. The eluate was reduced in a vacuum concentration system. The pellet was stored at $-20 \text{ }^\circ\text{C}$ before carbon isotope analysis.

The pellet kept for starch extraction in methanol was centrifuged (10 min, 2000 g, $4 \text{ }^\circ\text{C}$), the supernatant was discarded and the residue was dried for 15 min in a vacuum concentration system. The pellet was then suspended in 1 mL of HCl 6N. After vortexing and centrifugation (5 min, 2000 g, $4 \text{ }^\circ\text{C}$), the supernatant was retained and mixed with 8 mL of 60% methanol. Starch was then precipitated one day at $-20 \text{ }^\circ\text{C}$ and dried with the vacuum concentration system. Samples of starch were stored at $-20 \text{ }^\circ\text{C}$ before carbon isotope analysis. Extracted sugars and starch represented about 9% and 5% of the bulk leaf dry mass, respectively.

The carbon isotope composition of 1 mg of bulk leaf, soluble sugar or starch organic material was measured with a continuous flow isotope ratio mass spectrometer (Delta S; Thermo Finnigan, Bremen, Germany) coupled to an elemental analyser (Carlo Erba NA 1 500 NC, Rodano, Italy). The relative abundance of ^{13}C (‰) was expressed as (Craig, 1957) :

$$\delta^{13}\text{C} = (R_s - R_b)/R_b \times 1000$$

where R_s and R_b refer to the $^{13}\text{C}/^{12}\text{C}$ ratio in the sample and in the Pee Dee Belemnite standard, respectively.

Carbon isotope discrimination ($\Delta^{13}\text{C}$) was calculated as:

$$\Delta^{13}\text{C} \approx \delta^{13}\text{C}_{\text{air}} - \delta^{13}\text{C}_{\text{sample}}$$

where $\delta^{13}\text{C}_{\text{air}}$ is the carbon isotope composition of atmospheric CO_2 . $\delta^{13}\text{C}_{\text{air}}$ was assessed in the greenhouse by growing *Zea mays* plants, recording the $\delta^{13}\text{C}$ of leaf organic matter and using the procedure of Marino and McElroy (1991) to compute $\delta^{13}\text{C}_{\text{air}}$ which was found to be equal to $-9.80 \pm 0.23\text{‰}$ (average \pm standard deviation).

The modelled relationship between $\Delta^{13}\text{C}$ and W_i is (Farquhar and Richards, 1984) :

$$\Delta^{13}\text{C}_{\text{sample}} = b - (b - a) \frac{1.6 W_i}{c_a}$$

with: a , discrimination during diffusion of CO_2 through stomata (4.4‰) and b , discrimination during carboxylation of RuBP (27‰ taking into account 10% C fixed by PEPC; Farquhar and Richards, 1984) and c_a , atmospheric CO_2 mole fraction (continuously monitored in the greenhouse with a mean value of $394 \pm 12 \mu\text{mol mol}^{-1}$). The resulting equation is then $\Delta^{13}\text{C}_{\text{sample}} \approx 27 - 0.092W_i$.

2.3. Gas exchange measurements

Leaf gas exchange was recorded on one leaf per individual plant during July 2004, with an open flow gas exchange system (Li-6400, Li-Cor, Lincoln, NE, USA). Leaves were inserted into the chamber and photosynthesis was induced for about 40 min at 25 °C, at a CO_2 mole fraction of $390 \mu\text{mol mol}^{-1}$, a photosynthetic photon flux density of $1200 \mu\text{mol m}^{-2} \text{s}^{-1}$, and an air flux of $300 \mu\text{mol s}^{-1}$. Light saturated net CO_2 assimilation rate (A_{sat}) and stomatal conductance for water vapour (g_s) were recorded and intrinsic water use efficiency (W_i) was computed as the ratio between A_{sat} and g_s . Response curves of net assimilation rate (A) to CO_2 mole fraction in intercellular air spaces (c_i) were recorded with the following procedure: a decrease in CO_2 mole fraction from $390 \mu\text{mol mol}^{-1}$ to $50 \mu\text{mol mol}^{-1}$ in five steps. For each step, four min were allowed for gas exchange stabilization before measuring three times environmental parameters, A and estimating c_i . These curves were used to adjust maximal carboxylation rate of Rubisco (V_{cmax} ; von Caemmerer and Farquhar, 1981) as described by Dreyer et al. (2001). The adjusted model did not explicitly include internal conductance to CO_2 , and the computed values represent therefore apparent V_{cmax} .

2.4. Chlorophyll content

Relative chlorophyll content (in CCI unit) was estimated using a CCM-200 (Chlorophyll Content Meter, Opti-Sciences, Hudson, NH,

USA). Total chlorophyll content per leaf area (Chl) was derived using the following relationship calibrated for oak using biochemical chlorophyll extraction according to Barnes et al. (1992):

$$\text{Chl} (\text{g m}^{-2}) = 0.021 (\text{CCI unit}) + 0.05.$$

Total chlorophyll content was measured two times at different dates on three leaves per tree with three to five replicates per leaf. The data presented in this study are the least-square mean for each genotype calculated from an Analysis of Variance model taking into account the date effect.

2.5. Leaf nitrogen content and leaf mass-to-area ratio

Leaf mass-to-area ratio (LMA) and leaf nitrogen content (N_{area} , N_{mass}) were estimated for the leaves used for $\delta^{13}\text{C}$ analysis of bulk leaf organic matter. Area of fresh leaves was measured with a Leaf Area Meter (Delta-T Devices Ltd., Cambridge, UK), whereas mass was determined after drying and LMA was calculated as the ratio of leaf mass per area. N_{mass} was measured during isotopic analysis with the elemental analyser (Carlo Erba NA 1 500 NC, Rodano, Italy) that was coupled to the mass spectrometer. Leaf nitrogen content on an area basis (N_{area}) was calculated from N_{mass} times LMA.

2.6. Leaf anatomy

An additional three leaves were harvested for leaf anatomy. Over all 14 discs (1 cm^2) were punched from three leaves per vegetative copy of each genotype for measuring stomatal density and leaf anatomy and were immediately frozen in liquid nitrogen and stored at -80 °C . Microphotographs were made with a variable pressure scanning electron microscope (model 1450VP, Leo, Cambridge, UK, 20–30 Pa inside chamber, accelerating voltage 15 kV, working distance 12 mm) and analysed for stomatal density and stomatal dimensions using the Visilog software (Noesis, France).

One microphotograph of the abaxial epidermis (0.72 mm^2) of nine discs from three different leaves was used to estimate stomatal density (SD). On each of the microphotographs 50 stomata were sampled for measuring guard cell surface (SA) and length (SL). Stomatal area index (SAI ; Bruschi et al., 2003) was calculated as stomatal density \times stomatal length, which can be seen as a rough indicator of potential total stomatal opening per leaf surface.

On the five other discs (1 cm^2), four semi-thin cryo sections were photographed to measure total leaf thickness (TLT) and thickness of palisade (PMT), or spongy mesophyll (SMT), or upper and lower epidermal layers (UET and LET, respectively).

2.7. Statistical analyses

Using the whole data-set, correlations between traits were computed using STATISTICA software Version 7.1 (StatSoft, Inc. 2005). Correlations are based on genotype means and can therefore be seen as representing genetic differences among siblings within the family. Pearson's correlation coefficients were estimated with a correlation matrix or with regression analyses. As all correlations were based on the combination of two continuous variables, model II regression (geometric mean regression) was used to estimate the equation parameters (Sokal and Rohlf, 2000). All statistical tests were considered significant when $p < 0.05$.

Table I. Correlation coefficients (*R*) between genotype means of leaf traits (*N* = 12).

	$\Delta^{13}\text{C}_{\text{su}}$	$\Delta^{13}\text{C}_{\text{st}}$	$\Delta^{13}\text{C}_{\text{b}}$	W_i	A_{sat}	g_s	V_{cmax}	N_{mass}	N_{area}	Chl	<i>SD</i>	<i>SL</i>	<i>SA</i>	<i>SAI</i>	LMA	TLT	UET	LET	PMT	SMT	
$\Delta^{13}\text{C}_{\text{su}}$	*																				
$\Delta^{13}\text{C}_{\text{st}}$	0.93	*																			
$\Delta^{13}\text{C}_{\text{b}}$	0.76	0.90	*																		
W_i	-0.94	-0.87	-0.73	*																	
A_{sat}	0.55			-0.53	*																
g_s	0.72	0.56		-0.71	0.97	*															
V_{cmax}					0.91	0.83	*														
N_{mass}								*													
N_{area}								0.76	*												
Chl										*											
<i>SD</i>	0.52	0.53	0.68	-0.55	0.55	0.62					*										
<i>SL</i>	-0.57	-0.55	-0.59	0.67		-0.51					-0.62	*									
<i>SA</i>	-0.57	-0.55	-0.53	0.75								0.82	*								
<i>SAI</i>			0.61		0.51	0.56					0.97			*							
LMA	0.56			-0.51					0.55						*						
TLT									0.59						0.62	*					
UET								-0.50									*				
LET																		*			
PMT									0.61						0.52	0.96				*	
SMT									0.53						0.68	0.93				0.81	*

Significant correlations at 5% are in bold; non-significant correlations at 10% are noted in italic. See list of abbreviations for trait names.

Analyses of variance were done with the R 2.5.0 software (R Development Core Team, 2007) and effects were considered significant at $p < 0.05$. Comparisons with earlier results (Brendel et al., 2008) on $\delta^{13}\text{C}_{\text{bulk}}$ were analysed using the following model :

$$y_{ijk} = \mu + g_i + e_j + (ge)_{ij} + \varepsilon_{ijk}$$

where y_{lmn} is the variable of interest, μ the overall mean, g_i the genotype effect, e_j the environment effect, $(ge)_{ij}$ the genotype \times environment interaction and ε_{ikr} the residuals. Partial coefficients of determination (R^2) for the effects of this ANOVA were calculated as the sums of squares (SS) of the effect divided by total SS. This is a measure of the proportion of the total variation that is explained by one effect.

The difference of “low versus high” within “ Δ ” or “N” extremes (hereafter called phenotype effect) was analysed on each variable of interest with a linear mixed model (*nlme* R-package, Pinheiro et al., 2007) of the form:

$$y_{lmr} = \mu + \alpha_l + b_m + g_i + \varepsilon_{lir}$$

where y_{lmr} is the variable of interest, μ the overall mean, α_l the phenotype fixed effect, b_m the random block effect, g_i the fixed genotype effect and ε_{lir} the residuals.

Block effects were checked with a log likelihood ratio test between models with and without a random block effect. They were for all traits non-significant ($p > 0.05$) and excluded from the model.

Residuals were assumed to follow a joint normal distribution with zero means, zero covariances and variances either constant (homoscedastic model) or different among copies of each genotype (heteroscedastic model). Heteroscedasticity was checked with a log likelihood ratio test between homoscedastic and heteroscedastic models and the appropriate model was retained for each analysis.

3. RESULTS

3.1. Comparison with earlier results

An ANOVA using individual data (years 2000 and 2002) from Brendel et al. (2008) and $\Delta^{13}\text{C}_{\text{b}}$ from the present study showed significant effects ($p < 0.05$) for genotype (partial $R^2 = 39\%$), environment (37%) and genotype \times environment interaction (6.0%), explaining overall 82% of the observed variance.

3.2. Correlations among different measures of W_i

$\Delta^{13}\text{C}$ of the different metabolic compartments (bulk leaf, soluble sugars and starch) were all significantly correlated to W_i calculated from gas exchange measurements (Tab. I, Fig. 1). There was an improvement of the correlation from bulk leaf matter ($R^2 = 52.9\%$), over starch ($R^2 = 76.4\%$), to sugars ($R^2 = 87.6\%$). Slopes were similar between $\Delta^{13}\text{C}_{\text{su}}/W_i$ and $\Delta^{13}\text{C}_{\text{st}}/W_i$, whereas the intercept differed due to the isotopic shift from sugars to starch ($3.2 \pm 0.4\%$). The slope with bulk leaf matter differed from the two above, but the isotopic signal of sugars was closer to the overall bulk leaf matter (difference $0.5 \pm 0.7\%$).

The relationship of W_i with $\Delta^{13}\text{C}_{\text{su}}$ was similar to that predicted by the model (Farquhar and Richards, 1984), but had a significantly steeper slope ($p < 0.01$; ANCOVA), whereas the slopes for $\Delta^{13}\text{C}_{\text{st}}$ and $\Delta^{13}\text{C}_{\text{b}}$ did not differ from the model (dash line in Fig. 1). Regressions of W_i with carbon isotope

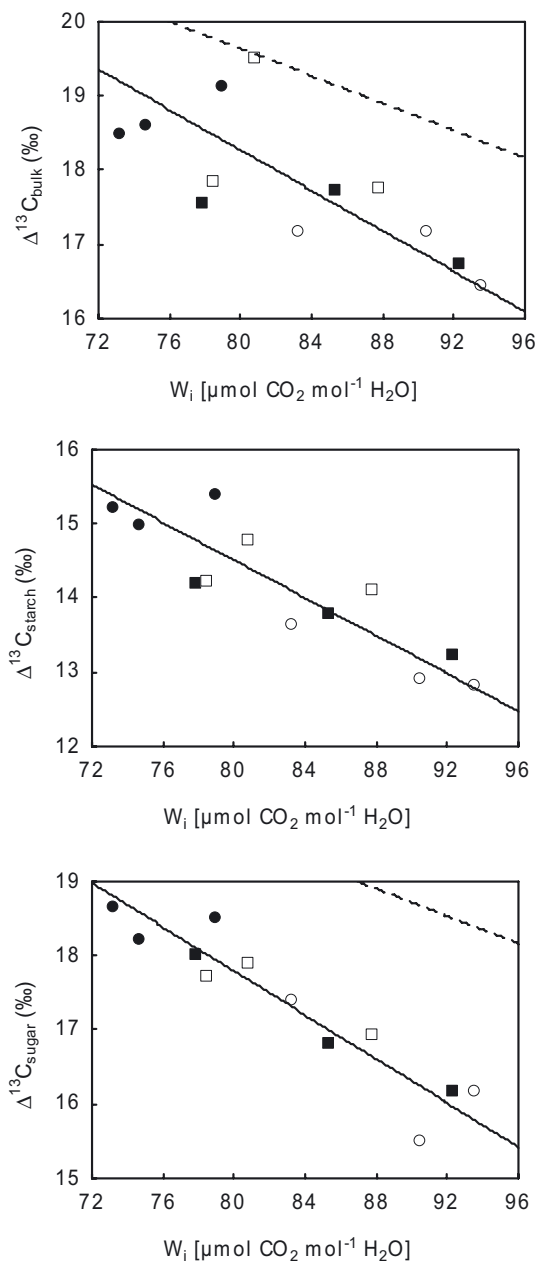


Figure 1. Relationship between genotype means of ^{13}C isotope discrimination (soluble sugars ($\Delta^{13}\text{C}_{\text{sugars}}$), starch ($\Delta^{13}\text{C}_{\text{starch}}$) and bulk matter ($\Delta^{13}\text{C}_{\text{bulk}}$) and W_i for the phenotypes “ Δ ” and “N”. Open circles represent genotypes with the phenotype “low Δ ”, open squares “low N”, closed circles “high Δ ”, closed squares “high N”. The dash line represents the theoretical relationships (Sect. 2.2.)

composition data of bulk leaf material recorded in the field by Brendel et al. (2008) were highly significant ($p < 0.005$), and R^2 was 0.77 ($N = 12$, data recorded during 2000), 0.74 ($N = 9$, data recorded during 2001) and 0.67 ($N = 12$, data recorded during 2002).

Genotypes with higher W_i had significantly lower stomatal conductance (Fig. 2a), and had also a tendency to lower

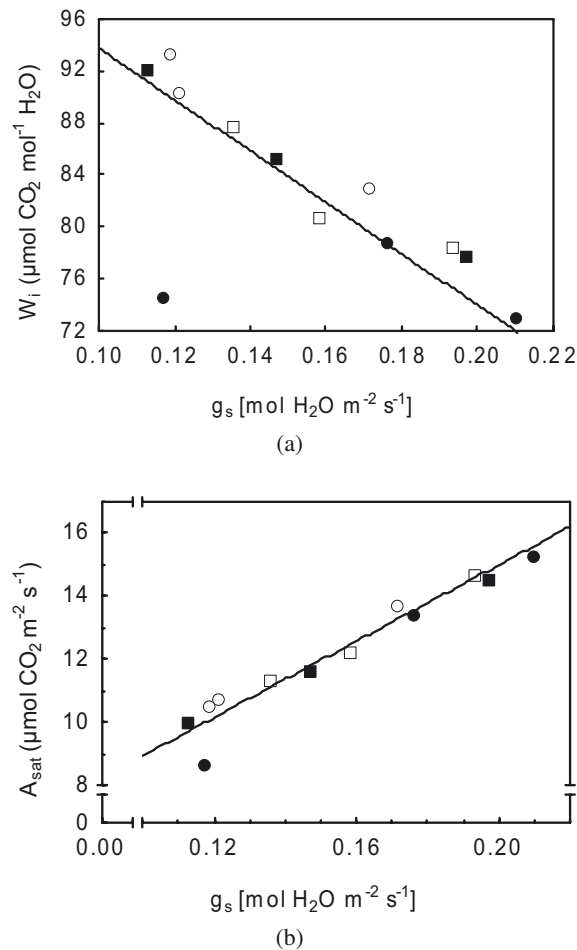


Figure 2. Relationship between stomatal conductance and (a) intrinsic water use efficiency (W_i) or (b) light saturated assimilation rate (A_{sat}). Each point represents the mean of a genotype. Symbols as in Figure 1. For the outlier in graph a, only one copy had been available for this genotype (170).

A_{sat} (Tab. I). Genotypes with higher A_{sat} showed higher values of V_{cmax} (Tab. I) and higher stomatal conductance (Fig. 2b). Traits related to photosynthesis such as chlorophyll content, leaf nitrogen content or V_{cmax} were not intercorrelated (Tab. I). However genotypes with higher stomatal conductance did also have an increased stomatal density (Tab. I) and a higher $\Delta^{13}\text{C}_b$ (Fig. 3). Differences among genotypes in stomatal anatomy were concurrent with differences in density such that genotypes with longer stomata (SL) had a lower stomatal density. Differences among genotypes in thickness of leaf parenchyma did not concur with differences in $\Delta^{13}\text{C}_b$, W_i or gas exchange parameters.

3.3. Differences in $\Delta^{13}\text{C}$ and associated leaf traits between extreme phenotypes

Genotypes for this study had been chosen because they displayed extreme phenotypes. Therefore, we compared

Table II. Genotype effect estimated for all data (*p*-level; explained variance) and means, standard deviations (*SD*) and differences (*p*-level) between extreme phenotypes “ Δ ” and “N” (see text for the definition of the phenotypes).

	Phenotypes Δ		Effect	Phenotypes N		Effect
	Mean \pm <i>SD</i>			Mean \pm <i>SD</i>		
	Low Δ	High Δ		Low N	High N	
$\Delta^{13}\text{C}_{\text{su}}$ (‰)	16.4 \pm 1.1	18.5 \pm 0.5	< 0.001	17.5 \pm 0.8	17.1 \pm 0.9	–
$\Delta^{13}\text{C}_{\text{st}}$ (‰)	13.1 \pm 0.6	15.3 \pm 0.4	< 0.001	14.4 \pm 0.5	13.8 \pm 0.6	< 0.01
$\Delta^{13}\text{C}_{\text{b}}$ (‰)	17.0 \pm 0.5	18.8 \pm 0.6	< 0.001	18.4 \pm 1.1	17.4 \pm 0.7	< 0.01
W_i ($\mu\text{mol mol}^{-1}$)	89.0 \pm 14.5	75.7 \pm 7.5	–	82.3 \pm 12.2	84.4 \pm 14.5	–
A_{sat} ($\mu\text{mol m}^{-2}\text{s}^{-1}$)	11.7 \pm 2.4	13.2 \pm 3.2	–	12.8 \pm 3.1	12.3 \pm 3.0	–
g_s ($\text{mol m}^{-2}\text{s}^{-1}$)	0.14 \pm 0.04	0.18 \pm 0.05	–	0.16 \pm 0.06	0.16 \pm 0.07	–
$V_{\text{c max}}$ ($\mu\text{mol m}^{-2}\text{s}^{-1}$)	76.2 \pm 12.0	78.1 \pm 18.0	–	75.4 \pm 15.8	80.2 \pm 14.2	–
N_{mass} (mg g^{-1})	32.3 \pm 3.2	32.1 \pm 3.0	–	29.9 \pm 4.3	34.8 \pm 3.9	0.01
N_{area} (g m^{-2})	2.78 \pm 0.39	3.02 \pm 0.23	–	2.67 \pm 0.44	2.96 \pm 0.28	0.04
Chl (g m^{-2})	0.93 \pm 0.16	0.95 \pm 0.09	–	0.96 \pm 0.11	0.98 \pm 0.06	–
<i>SD</i> (mm^{-2})	384 \pm 39	438 \pm 37	0.04	476 \pm 75	447 \pm 64	–
<i>SL</i> (μm)	24.1 \pm 1.1	23.5 \pm 0.7	–	22.8 \pm 0.7	23.7 \pm 0.8	< 0.001
<i>SA</i> (μm^{-2})	352 \pm 32	334 \pm 21	–	339 \pm 19	344 \pm 24	–
<i>SAI</i> (mm^{-1})	9.2 \pm 0.6	10.3 \pm 1.0	0.03	10.8 \pm 1.5	10.6 \pm 1.5	–
<i>LMA</i> (g m^{-2})	86.3 \pm 9.0	94.9 \pm 11.7	–	89.6 \pm 11.8	85.6 \pm 7.7	–
<i>TLT</i> (μm)	149 \pm 14	152 \pm 5	–	158 \pm 10	153 \pm 5	–
<i>UET</i> (μm)	10.6 \pm 0.6	10.7 \pm 0.6	–	10.6 \pm 0.8	10.5 \pm 1.0	–
<i>LET</i> (μm)	9.5 \pm 1.2	8.9 \pm 0.5	–	9.2 \pm 0.6	9.3 \pm 0.8	–
<i>PMT</i> (μm)	67.1 \pm 7.5	67.9 \pm 3.0	–	71.4 \pm 7.3	68.5 \pm 2.8	0.02
<i>SMT</i> (μm)	61.8 \pm 7.0	64.4 \pm 3.7	–	66.7 \pm 4.3	65.2 \pm 3.7	–

Significant *p* values (< 0.05) are shown in bold, “–” no significant difference. See list of abbreviations for trait names.

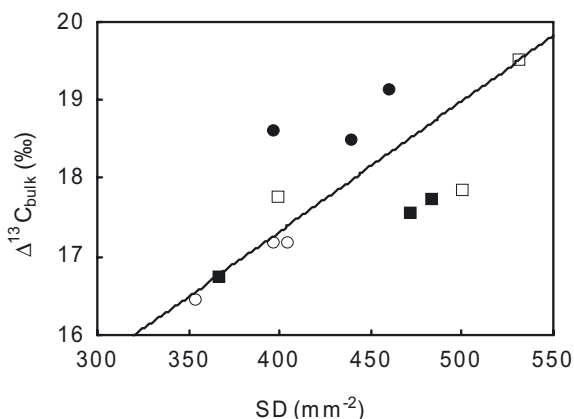


Figure 3. Relationship between $\Delta^{13}\text{C}$ of bulk leaf matter ($\Delta^{13}\text{C}_{\text{b}}$) and stomatal density (*SD*). Symbols as in Figure 1. Equation of the geometric mean correlation: $\Delta^{13}\text{C}_{\text{b}} = 10.6 + 0.02 \text{SD}$, $r = 0.68$, $p = 0.02$. Each point represents the mean of a genotype.

genotypes with high versus low phenotype values using an ANOVA. This is a more stringent test compared to the correlation analysis and results are presented in Table II.

Values of ^{13}C isotope discrimination of bulk, starch and soluble sugars ($\Delta^{13}\text{C}_{\text{b}}$, $\Delta^{13}\text{C}_{\text{st}}$ and $\Delta^{13}\text{C}_{\text{su}}$, respectively) differed significantly between the phenotypes “low Δ ” and “high Δ ”. Differences between the phenotypes “low N” and “high N” were significant for $\Delta^{13}\text{C}_{\text{b}}$ and $\Delta^{13}\text{C}_{\text{st}}$ only. The mean differences in $\Delta^{13}\text{C}$ correspond to a 6 to 13% difference in W_i between the extremes “N” and 26 to 30% between the extremes

“ Δ ”. Differences between “low N” and “high N” or “low Δ ” and “high Δ ” were not significant for intrinsic water use efficiency (W_i), light saturated net CO_2 assimilation rate at ambient CO_2 concentration (A_{sat}), stomatal conductance for water vapour (g_s), and apparent maximal carboxylation rate of Rubisco (V_{cmax}) (Tab. II).

The “high N” phenotypes displayed a consistently higher leaf N than “low N”, whereas there was no difference between “low Δ ” and “high Δ ” (Tab. II) for leaf nitrogen. Chlorophyll content (Chl) and leaf mass-to-area ratio (*LMA*) were similar among the phenotypes (Tab. II).

Among the measured leaf anatomy traits, only palisade mesophyll thickness (*PMT*) differed between “low N” and “high N”, the former being 2.9 μm thicker (Tab. II). Stomata were significantly longer in “high N” compared to “low N” (Tab. II) whereas stomatal density (*SD*) and stomatal area index (*SAI*) were significantly smaller for “low Δ ” compared to “high Δ ” (Tab. II).

4. DISCUSSION

4.1. Stability of genotypic differences for different measures of W_i

The tight correlations found between $\Delta^{13}\text{C}$ recorded on different metabolic compartments with varying turnover rates (bulk material, starch and soluble sugars), and therefore temporal integration scales (Brugnoli et al., 1988; Brugnoli and Farquhar, 2000), suggest that differences among genotypes

are robust with respect to these time-scales. The relationships found between temporally independent measurements of W_i and $\Delta^{13}\text{C}$ are close to the relationship predicted by the theoretical model (Farquhar and Richards, 1984), where $\Delta^{13}\text{C}$ of material with shorter temporal integration results in tighter correlation with W_i estimated from instantaneous gas exchange measurements (Fig. 1). It should be stressed that these relationships between W_i and $\Delta^{13}\text{C}$ were due to genetic differences among genotypes under common, optimal conditions and not due to acclimation of water use efficiency to different environmental conditions as observed in earlier studies (Guehl et al., 1994; Picon et al., 1996; Ponton et al., 2002). An observed genetic variability of $\Delta^{13}\text{C}$ can relate to a variability of W_i as well as to a variability of the parameters of the theoretical model linking these two traits. In fact, the correlation between $\Delta^{13}\text{C}$ and W_i observed by Brendel et al. (2008) was weak ($R^2 = 0.07$), suggesting that either a large part of the variability of $\Delta^{13}\text{C}$ was not related to W_i or that W_i from gas exchange measurements were estimated with much noise due to environmental influence. The here presented data, using more representative gas exchange measurements, showed that up to 87% of the genetic variability observed in $\Delta^{13}\text{C}$ can be related to W_i . Therefore, the closeness between observations and model affirms that measurement of carbon isotope discrimination on leaf bulk material in this pedunculate oak family (e.g. Brendel et al., 2008) reflected to a high degree variation in W_i .

“Low Δ ” and “high Δ ” phenotypes showed values for $\Delta^{13}\text{C}$ consistent with the sampling strategy, i.e., “low Δ ” displayed significantly lower values than “high Δ ”. When data from the present study and from Brendel et al. (2008) were compared, the relative differences among genotypes for $\Delta^{13}\text{C}$ were found to be largely independent from environmental conditions, that is there was very little genotype \times environment interaction. The relatively large overall environmental effect is probably due to the climate differences, where the field site in southern France was drier and hotter compared to the well-irrigated plants in the temperature controlled greenhouse. Thus, genotypic differences in $\Delta^{13}\text{C}$ were conserved among years, through contrasting environments and among different metabolic pools, which extends observations made by Johnsen et al. (1999) on *Picea mariana* and Lauteri et al. (1997) on *Castanea sativa*.

5. VARIABLES DRIVING VARIATION OF W_i WITHIN THIS PEDUNCULATE OAK FAMILY

W_i is calculated as the ratio between assimilation rate and stomatal conductance and therefore systematic variation of both traits among genotypes can change W_i . Furthermore, variation in A might be controlled by variation in g and vice versa. Here, variation detected among genotypes for light saturated g_s and A_{sat} suggest a strong control of g_s on A_{sat} and thus a major influence of g_s on differences in W_i . Differences in A_{sat} were representing differences in photosynthetic capacity as estimated by V_{cmax} , however, again, there was an overall correlation between V_{cmax} and g_s . Variation in leaf nitrogen and

chlorophyll content among genotypes did not have an effect on V_{cmax} , A_{sat} or on W_i . Also thickness of leaf parenchyma, which could theoretically have an impact on photosynthetic capacity, did not show any relationship with estimators of W_i . Overall, variation in traits related to photosynthetic capacity did not seem to be the driver for variation in any of the estimators of intrinsic water use efficiency.

Variation in W_i due to photosynthetic capacity had been studied specifically by using the selected “low N” and “high N” genotypes. This selection was effective for $\Delta^{13}\text{C}$ and leaf nitrogen content, as the expected differences were observed between the extreme phenotypes, whereas it was not effective for leaf chlorophyll content. This might be due to a low heritability of leaf chlorophyll. To our knowledge, heritability of chlorophyll content has not yet been estimated in a temperate forest tree species. Narrow sense heritability estimates for leaf chlorophyll content for other plant types rarely exceed 50% (e.g. Collaku et al., 2005; Kabanova et al., 2000 for wheat, Hervé et al., 2001 for sunflower), suggesting considerable environmental influence. This might be due to rapid (within days) acclimation to current irradiance, as shown by Frak et al. (2001) for *Juglans nigra x regia* seedlings. Additionally to the specific selection of “N” genotypes, the pedunculate oak saplings in the present study were raised with optimal water supply, which should reduce the stomatal limitation of assimilation rate and thus facilitate the expression of differences in photosynthetic capacity. However, “low N” and “high N” did not present difference in A_{sat} or in V_{cmax} , suggesting that the diversity of photosynthetic capacity might either not be well estimated by leaf N and chlorophyll content or not be a driver for variability in W_i within this family.

Otherwise, variation in stomatal conductance was a driver for the variation of W_i , as suggested by the strong correlation of g_s with W_i and $\Delta^{13}\text{C}_{\text{su}}$. Brendel et al. (2008) had not detected an correlation between $\delta^{13}\text{C}$ or W_i and g_s , probably due to the difficulties of measuring gas exchange in the field. Further, the differences among genotypes in g_s concur with differences in stomatal density (SD), i.e. genotypes with higher g_s also displayed more stomata per leaf surface. Differences in stomatal density were also related to differences in $\Delta^{13}\text{C}_b$, and a similar tendency was detected for other estimates of W_i . Few publications have analysed the relationship between $\Delta^{13}\text{C}$ and stomatal density among genotypes. A positive relationship between $\Delta^{13}\text{C}$ and stomatal density was also found for *Pinus ponderosa* provenances (Cregg et al., 2000) and among poplar clones from *Populus deltoides* \times *nigra* hybrids (Monclus et al., 2006), whereas Dillen et al. (2008) found a negative relationship for a *Populus deltoides* \times *trichocarpa* family. Genotypes with larger stomatal density showed higher g_s , which could lead to lower W_i . The model proposed by Nobel (1999) suggests a positive relationship between stomatal density and g_s . This was confirmed by Pearce et al. (2006) for several poplar species in a common garden and by Kundu and Tigerstedt (1999) for different provenances of *Azadirachta indica* in a greenhouse. A positive relationship between stomatal density and g_s would thus be the link for the observed positive correlation between $\Delta^{13}\text{C}$ and stomatal density, and could therefore

be a cause for the observed diversity in W_i among genotypes of this pedunculate oak family.

We also observed that genotypes with an increased stomatal density had smaller stomata (based on estimates of stomatal guard cell length SL). A similar relationship was observed by Hetherington and Woodward (2003) across different species but also within species. This relationship suggests a compensating effect resulting in a stable total stoma opening area. However our data suggest that this trade-off was only of limited extent as stomatal area index ($SAI = SL \times SD$), an estimator of total stoma opening area, still showed differences among genotypes related to differences in $\Delta^{13}C_b$. Aasamaa et al. (2001) have shown for tree species that the size of guard cells has an influence on stomatal aperture and thus on g_s . These results suggest a possible impact of stomatal density on variation in $\Delta^{13}C$ and thus W_i via a genetic diversity in maximum stomatal conductance within this family of pedunculate oak. According to Bergmann and Sack (2007), few gene products are required to regulate patterns of stomata in leaves. It has been shown in *Arabidopsis thaliana* that the gene *ERECTA*, which influences water use efficiency, can affect modulates mesophyll cell proliferation as well as stomatal density (Masle et al., 2005).

6. CONCLUSIONS

Using a relatively small number of siblings from a large pedunculate oak progeny, we showed that the diversity detected for $\Delta^{13}C$ in this progeny was closely related to instantaneous, intrinsic water use efficiency (W_i), as expected from the theoretical model linking these two traits (Farquhar and Richards, 1984). Genetic variability of $\Delta^{13}C$ and W_i was largely independent from different temporal integration scales and from different environments. We were not able to substantiate the influence of photosynthetic capacity on W_i or $\Delta^{13}C$ that had been suggested by the co-localisation of QTL for $\Delta^{13}C$, nitrogen and chlorophyll content. However, the large genetic variation in stomatal density and stomatal area index, as well as the tight correlation between stomatal conductance and $\Delta^{13}C$ suggest that differences in water use efficiency within this pedunculate oak family might be due to differences in stomatal conductance.

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