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Stomatal density in *Quercus petraea* and *Q. robur* natural populations in Northern Turkey

Abstract: This study examined variation of stomatal density in two populations of *Quercus petraea* (Matt.) Liebl. and two populations of *Q. robur* L. from northwestern Turkey. Stomatal density was determined in fully expanded and dried leaf samples that were collected from trees under natural conditions. Stomatal densities of *Q. petraea* and *Q. robur* varied from 186 to 459 per mm^2 (mean value: 333 stomata per mm^2) and from 397 to 826 per mm^2 (mean value: 517 stomata per mm^2), respectively. Significant differences in stomatal density were found between these two oak species in Turkey as well as between populations within species. Strong and significant negative correlations were observed between stomatal density and leaf length within each species and across the species. While in Central Europe *Q. petraea* occurs in drier environments than *Q. robur*, in the present study *Q. robur* populations grow in more arid environments and have smaller leaves and a higher stomatal density than *Q. petraea*. Stomatal density had negative correlations with each of the other leaf characters apart from sinus width. In addition, the interspecific P_{ST} value (0.48) for stomatal density was relatively high compared to the mean genetic differentiation calculated at eight microsatellite loci (F_{ST} = 0.030), suggesting different local adaptations of populations. Further studies that include additional populations will be necessary to associate genetic variation at candidate genes with phenotypic and environmental variation.

Keywords: stomatal density, genetic differentiation, phenotypic differentiation

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Introduction

Both genetic and plastic factors account for changes in stomatal development. Phenotypic plasticity is influenced by several environmental factors such as temperature, light intensity, water availability and atmospheric levels of carbon dioxide (Bradshaw & Hardwick, 1989; Sultan, 2000). Stomatal density is a suitable phenotypic characteristic for understanding the long-term adaptation and short-term response of plant species to changing environmental conditions at large spatial scales (Hetherington & Woodward, 2003; Bresson et al., 2011; Wang et al., 2014). Furthermore, studies exploring stomatal characteristics

such as stomatal density may give information about the degree to which plants that are grown outside their natural habitats adapt to new environmental conditions (Sevik et al., 2017). For example, Batos et al. (2010) stated that the low number of stomata detected for *Quercus robur* in Northern Serbia (Bojčinska šuma) could be a result of species adaptation to increased air pollution, since the locality is situated in the proximity of a thermoelectric power plant. Stomatal density has shown significant variation within individuals, cultivars or ecotypes of a single species, as well as within plant communities (Jones, 2014).

Pedunculate oak (*Quercus robur* L.) and sessile oak (*Q. petraea* (Matt.) Liebl.) are two sympatric, widespread tree species with high economic and ecological importance that cover most of Europe from Spain to Russia, and from Scotland to Turkey (Bodénès et al., 1997). Both species are supposed to have different ecological requirements; in Central and Western Europe, *Q. petraea* is more drought tolerant and is generally found on better drained soils, while *Q. robur* is usually found on nutrient rich, heavy soils prone to temporary water-logging (Breda et al., 1993; Aas, 1998; Bruschi, 2010; Zanetto et al., 1996).

In Turkey, both *Q. robur* and *Q. petraea* occur in similar semi-humid to arid climates (Ugurlu et al., 2012), suggesting different local adaptations of these species in Turkey compared to the European species distribution range. Based on the precipitation effectiveness index (Erinç, 1965), Yücedağ & Gailing (2013) reported that *Q. petraea* populations were located in semi-humid conditions and *Q. robur*

populations in semi-arid to arid climates. Likewise, Eaton et al. (2016) pointed out that these oak species show phenotypic differences in conditions far from their optimum. The general tendency is for *Q. robur* to grow in more continental climates, while the more drought tolerant *Q. petraea* prefers to grow in more Atlantic climates (Eaton et al., 2016).

Up to now, no studies have been performed on the variation of stomatal density and associations with leaf morphological characters in natural populations of *Q. petraea* and *Q. robur* in Turkey. The goals of the present study are (1) to assess differences in stomatal densities between species and between populations within species for two populations of *Q. robur* and two populations of *Q. petraea* in northwestern Turkey, (2) to analyze associations of stomatal density with seven leaf morphological characters obtained from a previous study (Yücedağ & Gailing, 2013), and (3) to calculate Phenotypic Trait Differentiation (P_{ST}) between species and between populations within species for stomatal density and seven leaf morphological characters.

Material and Methods

From their natural distribution in Turkey, four populations separated by a distance of about 50 km were sampled (Table 1; Fig. 1). Species were identified in the field using the taxonomic key by Aas (1993). Each population was represented by 50 individuals. Adult trees were about 30–70 years old, 15–30 cm in

Table 1. Basic characteristics of four *Quercus petraea* and *Q. robur* populations

Species	Population	Elevation (m)	Latitude	Longitude	Precipitation Effectiveness Index*	Climate	Soil
Q. petraea	Bartın-Abdipaşa	173	41°31'04"	32°32'42"	30.7	Semi-humid	Loam clay
	Zonguldak-Devrek	364	41°07'23"	31°59'58"	37.3	Semi-humid	Clay loam
Q. robur	Kastamonu-Daday	915	41°27'52"	33°20'48"	16.2	Semiarid	Sandy loam
	Karabük-Eflani	936	41°28'41"	32°51'54"	13.8	Semiarid	Sandy loam

*Precipitation Effectiveness Index was calculated from 41 annual (1970–2011) climate records (Anonymous, 2013). This index was calculated by taking the ratio of total annual precipitation to the annual mean maximum temperature (Erinç, 1965).
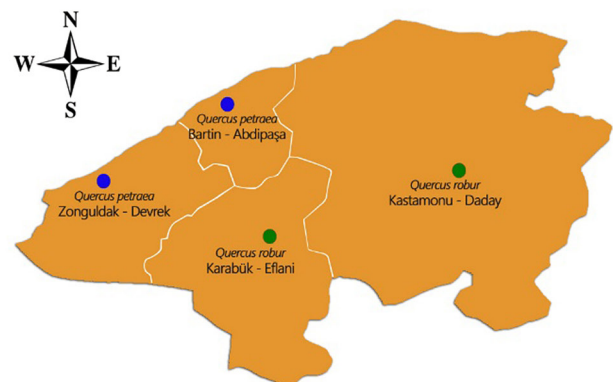

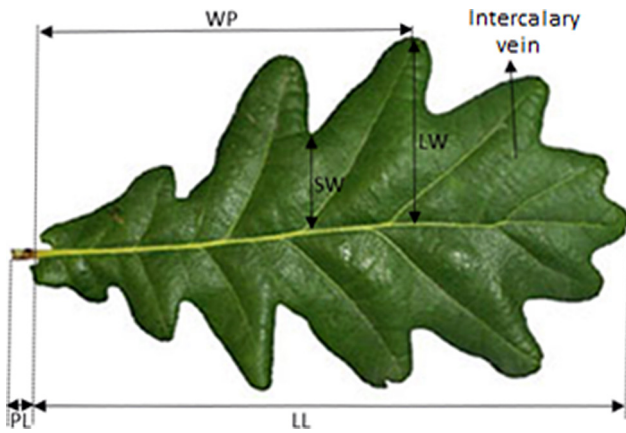Fig. 1. Geographical locations of the studied oak populations

Fig. 2 Five directly measured variables and intercalary vein

diameter at breast height (DBH), and originated from natural regeneration. Five fresh and fully expanded shade leaves from the first flush of the year were collected in the mid-to-upper crown of the trees. Leaf samples were dried with silica gel in zip-lock plastic bags for two weeks and then stored in paper bags.

In a previous study, five leaves from each of the 200 trees were measured for biometrical verification in each population at seven leaf morphological characters (Yücedağ & Gailing, 2013). The variables comprised five directly measured (Fig. 2), and two counted characters according to the methods used previously in Aas (1993) and Kremer et al. (2002). The following characters were assessed: 1) lamina length (LL), 2) petiole length (PL), 3) length of the widest lobe (LW), 4) sinus width (SW), 5) length of lamina at the largest width (WP), 6) number of lobes (NL), 7) number of intercalary veins (NV). All lobes except the terminal lobe were counted. Intercalary veins are counted if they range at least half way from the midrib to the base of the sinus (Yücedağ & Gailing, 2013). These leaf morphometric data were reanalyzed to explore phenotypic differentiation between species and trait correlations with stomatal density.

Stomatal density was determined on dried leaves that had been used previously for the leaf morphological measurements. Specifically, one leaf per tree was used for stomata counts for 48 trees from Abdipaşa, 49 trees from Devrek, 44 trees from Daday and 49 trees from Eflani in 2017. Slides were prepared for stomatal counts as follows: Each leaf was covered with a thin layer of clear fingernail polish in the intercostal zone between two third-order veins of a central lobe on the lower epidermis (area of high photosynthetic activity). Next, a short strip of clear tape was used to transfer the stomatal imprint to a microscope slide, and the number of stomata was counted on photomicrographs (×200 magnification) in a fixed leaf area (0.1938 mm²) using a stage micrometer of the Nikon Eclipse E 400 Nush-baimine Leica microsystems.

An ANOVA was performed to test for significant differences in trait mean values between species and between populations within species. In this one-way variance analysis, populations were considered as fixed variables. Furthermore, the mean values and standard errors of the mean for stomatal density for each of the populations were calculated. The relationship between stomatal density and each of the seven leaf traits (see Supplementary Table 1) was analyzed using Pearson correlations. Analogous to Quantitative Trait Differentiation (Q_{ST}, Spitze, 1993), phenotypic variance was estimated for stomatal density and as comparison for leaf traits as:

$$P_{ST} = \sigma^2_{G(among)}/(\sigma_{G(among)} + 2\sigma^2_{G(within)}),$$

with $\sigma^2_{G(among)}$ being the variance between species/ populations and $\sigma^2_{G(within)}$ the variance within species/ populations (Raeymaekers et al., 2007; Pujol et al., 2008). P_{ST} values which are magnitude higher genetic differentiation at neutral genetic markers (F_{ST} values) can be a signature of selection (Raeymaekers et al., 2007; Brommer, 2011). Additionally, Phenotypic Trait Differentiation (P_{ST}) between species estimated in the present study was compared with the mean genetic differentiation values (F_{ST} values) observed at eight microsatellite loci for the same populations (Yücedağ & Gailing, 2013). ANOVA, variance components and Pearson correlation analyses were conducted with the SPSS program (SPSS Inc., 2011).

Results

Mean stomatal densities of *Q. petraea* and *Q. robur* were 333 and 517 per mm² and varied from 186 to 459 per mm² and from 397 to 826 per mm², respectively. The analysis of variance revealed significant differences between species at the 0.001 probability

Table 2. Range, mean, F ratio and P_{ST} values for stomatal density by population/species

Species	Population	Sample size	Stomatal Density (per m²)				
			Range		Mean	F ratio	P	P_{ST}
Q. petraea	Bartin-Abdipaşa	48	186–459	186–459	333±1.4	7.25	<0.01	0.26
	Zonguldak-Devrek	49	256–459					
Q. robur	Kastamonu-Daday	44	397–826	397–826	517±2.7	16.92	<0.001	0.33
	Karabük-Eflani	49	408–562					
Between Species						351.51	<0.001	0.48

Table 3. Correlation coefficient between leaf characters and stomatal density, and P_{ST} values for seven leaf characters

Leaf characters	Abbreviations	Correlation coefficient	P_{ST} values		
			between populations within *Q. petraea*	between populations within *Q. robur*	between species
Lamina length	LL	−0.65**	0.21	0.28	0.42
Petiole length	PL	−0.36**	0.14	0.19	0.16
Length of the widest lobe	LW	−0.57**	0.18	0.24	0.23
Sinus width	SW	0.03ns	0.01	0.04	0.08
Length of lamina at the largest width	WP	−0.53**	0.11	0.17	0.22
Number of lobes	NL	−0.57**	0.10	0.17	0.20
Number of intercalary veins	NV	−0.22*	0.22	0.26	0.38

** $p < 0.001$, * $p < 0.01$, ns: not significant.

level for stomatal density ($P_{ST} = 0.48$). Significant differences were also found between populations within *Q. petraea* ($P_{ST} = 0.26$) and between populations within *Q. robur* ($P_{ST} = 0.33$) (Table 2). As seen in Table 3, Pearson correlation coefficients revealed a negative correlation between stomatal density and all leaf characters apart from SW ($p = 0.675$). The highest coefficient of correlation (−0.65) was observed between stomatal density and LL. Differentiation at phenotypic traits (P_{ST}) for leaf characters ranged from 8% to 42% between species, from 1% to 22% between populations within *Q. petraea* and from 4% to 28% between populations The individual population means and standard error bars are shown in Fig. 3 with the populations of *Q. robur* showing a significantly higher stomatal density (per mm^2) than *Q. petraea* populations. On the other hand, *Q. petraea* populations having a higher precipitation effectiveness index showed lower stomatal densities than *Q. robur* populations (Fig. 3).

Fig. 3 Means and standard deviations for stomatal density across the four populations of *Q. petraea and Q. robur*. Groups A and B are significantly different ($P < 0.001$)

Discussion

There are large differences in stomatal density across land plant lineages (Brodribb & McAdam, 2017). Smaller stomata provide more sites for fast gas exchange through stomata and thus an increased potential for carbon dioxide uptake when water availability is low (Bruschi et al., 2000).

The mean stomatal density in *Q. robur* populations from northwestern Turkey (490 and 548 stomata per mm^2) was much higher than that of *Q. petraea* populations (316 and 349 stomata per mm^2) (see Fig. 3). In contrast, in European populations, *Q. petraea* revealed a higher stomatal density than *Q. robur* (Bruschi, 2010; Batos et al., 2010). Likewise, *Q. petraea* seedlings from Germany showed a higher stomatal density than *Q. robur* under common garden conditions (unpublished results). The mean stomatal density (381 per mm^2) for Italian *Q. petraea* populations reported by Bruschi (2010) was similar to our observations in northwestern Turkey. The reason for this similarity can be that the values of mean annual temperature and rainfall are similar in both regions. However, in *Q. robur* populations from northern Serbia, mean stomatal density was much lower than in Turkish populations and varied from 256 to 353 per mm^2 (Batos et al., 2010). The relatively low stomatal density in some *Q. robur* populations analyzed by Batos et al. (2010) may be related to their occurrence in more humid riparian areas. In addition, low stomatal density detected for *Q. robur* in populations from Northern Serbia could be an adaptation to the increasing air pollution, since the locality is situated in the proximity of the thermoelectric power plant.

In contrast to European populations (Saintagne et al., 2004; Bruschi, 2010; Annighöfer et al., 2015), *Q. robur* grows in comparatively dry environments in Turkey. Specifically, *Q. robur* populations of northwestern Turkey used in this study were found on semi-arid to arid sites while *Q. petraea* populations were located on semi-humid sites (Yücedağ & Gailing, 2013). Furthermore, *Q. petraea* populations of northwestern Turkey were at lower elevation than *Q.*
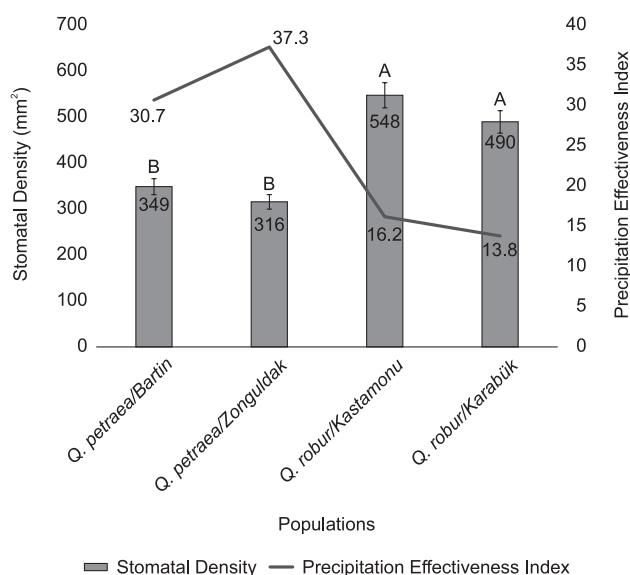
robur populations. In this study, it was found that stomatal density was higher in drier environmental conditions. Similar patterns were noticed also in other species such as the South African shrub *Protea repens* (Carlson et al., 2016) and European beech (Stojnic et al., 2015).

Genetic marker analyses using nuclear mircrosatellites revealed different patterns of interspecific differentiation between *Q. robur* and *Q. petraea* in Turkey than in Central Europe (Scotti-Saintagne et al., 2004; Yücedağ & Gailing, 2013). For example, earlier microsatellite analyses also revealed that interspecific differentiation at the outlier locus ssrQrZAG96 in European *Q. petraea* and *Q. robur* populations was higher than in the Turkish populations (Yücedağ & Gailing, 2013). These results point to different local adaptations in *Q. robur* and *Q. petraea* in Turkey than across the sympatric range in Central and Western Europe.

Specifically, strong and significant negative correlations were observed between stomatal density and leaf length for both species. Similarly, Pyakurel & Wand (2014) found that paper birch (*Betula papyrifera* Marsh) populations collected across Canada with larger leaf area had lower stomatal density. Stomatal density in the red mangrove (*Rhizophora mangle*) is inversely related to leaf area, especially leaf width (Peel et al., 2017). The smaller leaves of *Toona ciliata*, a forest tree in the mahogany family, had higher stomatal density under high air vapour pressure difference (Carins Murphy et al., 2014). Stomatal density may increase with decreasing leaf size to overcome a simple geometric practicality of fitting enough functional stomatal units per unit of leaf surface area to meet the desired CO_2 flux and to service photosynthetic capacity (Franks & Farquhar, 2007). These leaf characteristics provide a structural basis for the reduction of water loss through leaves, reducing over-transpiration and increasing water use efficiency. Plants with higher stomatal density can better adjust stomatal conductance to leaf water status (Hetherington & Woodward, 2003) suggesting that small leaves with high stomatal density are adaptations to dry environments. In addition, studies relating to leaf morphology and stomata are valuable for identifying ecologically important traits that can then be further analyzed in other experiments (Pyakurel & Wang, 2014). Stomatal densities can be influenced by a number of external factors including temperature, light, humidity and CO_2 concentration (Lawson et al., 2014). However, identifying which factors have the greatest influence on plasticity in stomatal traits occurring in response to environmental factors was often difficult (Hill et al., 2015).

Species-specific responses to different environmental factors illustrate that the drivers of genetic and phenotypic differentiation can strongly differ even among related species distributed in similar landscapes (Riordan et al., 2016). Relatively high phenotypic differentiation between species for stomatal density ($P_{ST} = 0.48$) can be due to the combined effect of genetic and environmental differences. Differentiation between species at phenotypic traits (P_{ST}) for leaf characters ranged from 8% to 42%. Similarly, high Q_{ST} values for shoot height (0.70), root collar diameter (0.44), twig thickness (0.70) and leaf number (0.71) were determined in a common garden trial of *Q. macranthera* subsp. *syspirensis,* sampled from 12 populations in the northern part of Turkey (Yücedağ et al., 2018). The P_{ST} value (0.48) for stomatal density in the present study was considerably higher than the mean genetic differentiation values between *Q. petraea* and *Q. robur* observed at eight microsatellite loci ($F_{ST} = 0.030$). Higher differentiation at phenotypic traits than at putatively neutral nuclear microsatellites suggests that these traits are under divergent selection and involved in local adaptation (e.g., Conner & Hartl, 2004; Pujol et al., 2008; Whitlock, 2008; Brommer, 2011). P_{ST} is generally higher than neutral genetic differentiation, implying that natural selection overcomes ongoing gene flow to maintain morphological differences (Leinonen et al., 2013). Traits with high population variation may be considered to be more important for adaptation (St Clair et al., 2013). Phenotypic and genetic differentiation along environmental gradients, or across contrasting habitat types, can also be indicative of local adaptation (Savolainen et al., 2013). For instance, differentiation between regions for stomatal density and size as well as leaf size in *Arabidopsis thaliana* was stronger than expected from genome-wide patterns of diversity, suggesting local adaptation (Dittberner et al., 2018).

Conclusions

The current study revealed that differentiation in stomatal density is much higher between species than between different populations within species pointing to genetic differences between species in this region. *Quercus robur* populations on semi-arid sites in northwestern Turkey had much greater stomatal densities than European populations suggesting different adaptations to drought across the species distribution range. These results are of great ecological importance for understanding acclimation of different types of oaks. Further studies with additional populations should be carried out to associate genetic variation in candidate genes with phenotypic and environmental variation. Different genotype-environment interactions in different populations can result in different responses, relying on both genetic traits and the environment.

Acknowledgements

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