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ORIGINAL ARTICLE

Leaf morphological and genetic differentiation between *Quercus rubra* L. and *Q. ellipsoidalis* E.J. Hill populations in contrasting environments

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Abstract Hybridization is considered to play an important role in speciation and evolution. Given the predicted northward tree migration in the eastern USA due to the impact of climate change, hybridization between related species is expected to become more frequent due to overlapping distribution ranges in the future. Oak species are "hot spots" of contemporary hybridization, serving as model organisms in the development of ecological species concepts. *Q. rubra* L. and *Q. ellipsoidalis* E.J. Hill were selected as study species, since they show different ecological requirements but hybridize with each other where both species co-occur. To identify morphological species and differentiation patterns in this species pair in ten populations on the Upper Peninsula of Michigan we investigated both leaf morphological variation, and genetic variation at highly variable microsatellite markers. Cluster analyses using leaf morphological characters revealed two distinct clusters for directly measured leaf characters and three clusters when additionally leaf shape characters were considered. Two populations growing on dry and sandy sites and identified as *Q. ellipsoidalis* in the field and by genetic assignment analyses were differentiated from the other eight populations at leaf morphological characters. Strong and significant correlations of leaf morphological differences with genetic distances at microsatellite markers but not with geographic distances are consistent with a pattern of isolation by adaptation. Differentiation at genetic and leaf morphological characters between neighboring populations in contrasting environments suggested reproductive isolation between populations of different species, possibly as the result of divergent selection. More extensive sampling along the distribution range of both species and reciprocal transplant experiments between parental environments are necessary to better understand the role of interspecific gene flow and selection in the maintenance of species identity in red oak species (*Quercus* section Lobatae).

Keywords *Quercus rubra* · *Q. ellipsoidalis* · Hybridization · Leaf morphology · EST–SSRs

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Introduction

Quercus species are ecologically and economically important components of North American forests (e.g., McShea et al. 2007; Aldrich and Cavender-Bares 2011). Climate change is expected to enhance the competitiveness of drought-adapted species such as oaks in the northern USA and Canada (Woodall et al. 2009), and disjunct oak populations at the northern edge of their range are likely very important reservoirs of genetic variation needed for adaptation of these species to climate change.

Species boundaries among oaks are often not clear-cut due to the propensity of oaks to hybridize with related species of the same section. Thus, genetic differentiation at most genetic markers and morphological characters is comparatively low between hybridizing oak species (e.g., Hokanson et al. 1993; Mariette et al. 2002). Hybridizing oak species often occur in one stand within the range of gene flow but in contrasting environments, and hybridization between ecologically divergent species can be quite

frequent in mixed stands of European and North American oaks (Bacilieri et al. 1996; Curtu et al. 2009; Dodd and Afzal-Rafii 2004; Finkeldey 2000). Despite a considerable level of gene flow among species, genetic and morphological species identity has been maintained in the area of sympatry in species-rich European oak stands (Curtu et al. 2007, 2009). Hybrid individuals were more common among offspring (seeds) than among adult trees and occurred in the contact zones between species in intermediate environments, suggesting that selection on specific genes maintains adaptive species differences (Curtu et al. 2009). Similar studies have been carried out with North American oaks, also pointing to an important role of environmental selection in determining population genetic structure and species identity (Craft et al. 2002; Dodd and Afzal-Rafii 2004).

While oaks served as model organisms in the development of ecological species concepts (Van Valen 1976), the role of hybridization in adaptation to changing environments is poorly understood. Furthermore, the lack of comprehensive genetic and morphological data for many species pairs makes it difficult to circumscribe species boundaries and to distinguish between effects of phenotypic plasticity and genetic differences between populations with different phenotypes.

Quercus rubra L. (northern red oak) and *Q. ellipsoidalis* E.J. Hill (Hill's oak) offer a good model to study hybridization and local genetic adaptation, since they have a widely overlapping sympatric distribution in the Upper Great Lakes region but grow in different microenvironments. *Q. rubra* is common on mesic slopes and well-drained uplands, while *Q. ellipsoidalis* prefers drier sites and is described as the most drought-tolerant red oak species maintaining high photosynthesis rates at low leaf water potentials during drought (Abrams 1988, 1990).

Q. rubra is the dominant oak species at the northern edge of the species distribution. It shows a wide geographic distribution in eastern North America ranging from southern Ontario and northern Michigan in the north to Alabama in the south, growing under a wide range of climatic and edaphic conditions (Nixon 1997). It appears to hybridize with related red oak species within its range, including *Q. ellipsoidalis* (Jensen et al. 1993), *Q. coccinea* and *Q. velutina* (Hipp and Weber 2008; Voss 1985). *Q. ellipsoidalis* shows a more scattered distribution on drier sites at its northern distribution edge. The species is distinguished from *Q. rubra* by leaf and cup morphological characters and by its shrubby growth habit (Barnes and Wagner 2004; Hipp and Weber 2008) and by nuclear and genic microsatellite markers (Lind and Gailing, unpublished data). Due to high variability in morphological characters and the absence of morphological and genetic diagnostic characters, it is difficult to distinguish between these closely related species at morphological traits or at a limited set of genetic markers. Thus, earlier studies of this species pair on the Apostle Islands in Wisconsin revealed continuous, clinal variation in leaf morphological characters and low genetic differentiation between populations, interpreted as the result of interspecific hybridization (Hokanson et al. 1993; Jensen et al. 1993). Likewise, we observed a wide variation in leaf morphological characters on the Upper Peninsula of Michigan within populations but also pronounced differences in leaf size and growth habit between neighboring populations in different microenvironments (dry/mesic). We initially classified the populations on the very dry sites as *Q. ellipsoidalis* based on their shrubby growth habit, their small leaves, and acorn characteristics. Genetic marker analyses at nuclear and gene-based microsatellite markers confirmed the field assignment and showed a clear separation between *Q. ellipsoidalis* populations on dry sites and *Q. rubra* populations on mesic sites. Other populations with shrubby growth habit and small leaves on granite rock outcrops were identified as *Q. rubra* based on the genetic marker analyses (Lind and Gailing, unpublished data).

To explain the large, apparently continuous leaf morphological variation in *Q. rubra* and *Q. ellipsoidalis* populations, we developed two working hypotheses: (1) This variation is the product of phenotypic differences between two hybridizing species with incomplete reproductive isolation, or (2) it is the product of high phenotypic variation within a single species for example in response to the microenvironment (phenotypic plasticity) and/or as the result of genetic variation for leaf morphology within species. In order to distinguish between these alternative hypotheses, individual samples were assigned to distinct groups (species) based on leaf morphological differences and the results of the morphological assignment were compared with the genetic assignment based on allele frequency differences at highly variable microsatellite markers (Lind and Gailing, unpublished data) using the program Structure (Pritchard et al. 2000). If the high leaf morphological variation within populations is mainly caused by hybridization between phenotypically different species, we would expect low within-species leaf morphological variation, hybridization to be relatively frequent, and first and later generation hybrids within populations to have different intermediate leaf morphologies.

Comparatively high genetic differentiation at microsatellite markers between neighboring *Q. rubra* and *Q. ellipsoidalis* populations in contrasting microenvironments (mesic/dry) (Lind and Gailing, unpublished data) was consistent with a pattern of "isolation by adaptation" as the result of divergent selection (Nosil et al. 2009). In the present study we test whether there is a positive correlation between leaf morphological differences (e.g., difference in

leaf size and shape as adaptation to drought) and genetic differences at microsatellite markers which would support the "isolation by distance" hypothesis.

Materials and methods

Study sites

Five to ten sun leaves were sampled from a total of 434 trees representing ten stands in four regions of the Upper Peninsula of Michigan (Table 1, Fig. 1). The sites differed with regard to soil characteristics and forest type (Table 1). An especially strong contrast in soil types and growth forms was observed between neighboring populations in the Baraga Plains region (Table 1), with *Q. ellipsoidalis* (stands FC-C and FC-E) growing in low density in an open savanna together with scattered *Pinus banksiana* trees on very dry sites comprising deep outwash sands in a forest type characterized as Pine Barrens (Albert and Comer 2008). Additionally, *Q. ellipsoidalis* populations on the very dry site showed strong insect herbivory and very low seed production since 2009.

Q. ellipsoidalis stands FC-C and FC-E and *Q. rubra* stands HMR-MI, HMR-LP, and BR (granite rock outcrops) showed a shrubby growth habit. *Q. rubra* stands on the other sites were more typical of the red oak growth form. Genetic assignment analyses including *Q. rubra* and *Q. ellipsoidalis* reference samples (Hipp and Weber 2008) identified most individuals in *Q. rubra* populations as *Q. rubra* or *Q. rubra* introgressive forms, and most individuals in the two *Q. ellipsoidalis* populations as *Q. ellipsoidalis* or *Q. ellipsoidalis* introgressive forms (Lind and Gailing, unpublished data). The number of F_1 hybrids was low and varied from 0 to

Table 1 Sample locations and site characteristics of 10 natural populations of *Quercus rubra* and *Q. ellipsoidalis*

Name	Region	Population	Drainage class, soil type	Dominating species	Forest type	Latitude	Longitude	Altitude (m a.s.l.)
HMR-IH	Huron Mountain Reserve	Ives Hill	2, Peshekee-Rock outcrop	*Quercus*, *Acer*, *Pinus*	Sugar Maple-Hemlock forest	46°51′12.884″	87°50′42.824″	257
HMR-LI	Huron Mountain Reserve	Ives Lake	2, Peshekee-Rock outcrop	*Quercus*, *Acer*, *Pinus*	Sugar Maple-Hemlock forest	46°50′39.462″	87°51′17.978″	246
HMR-LP	Huron Mountain Reserve	Lily Pond	2, Peshekee-Rock outcrop	*Quercus*, *Acer*, *Pinus*	Sugar Maple-Hemlock forest	46°50′59.813″	87°49′48.806″	307
HMR-MI	Huron Mountain Reserve	Mount Ives	2, Peshekee-Rock outcrop	*Quercus*, *Acer*, *Pinus*	Sugar Maple-Hemlock forest	46°51′20.783″	87°51′24.026″	297
FC-A	Baraga County	Ford Center-A	3, Munising and Yalmer loamy sand	*Quercus*, *Acer*, *Tsuga*	Sugar Maple-Hemlock forest	46°39′9.407″	88°30′6.962″	423
FC-B	Baraga County	Ford Center-B	3, Munising and Yalmer loamy sand	*Quercus*, *Acer*, *Tsuga*	Sugar Maple-Hemlock forest	46°40′27.937″	88°31′27.397″	394
FC-C[a]	Baraga County	Ford Center-C	1, Grayling sand	*Pinus banksiana*, *Quercus*	Pine Barrens	46°39′14.454″	88°35′25.616″	398
FC-E[a]	Baraga County	Ford Center-E	1, Grayling sand	*Pinus banksiana*, *Quercus*	Pine Barrens	46°39′55.879″	88°33′19.775″	407
BR1	Brockway Mountain	Brockway	2, Arcadian-Michigamme-Rock outcrop	*Quercus*, *Betula*, *Pinus*	Aspen-Birch forests	47°27′57.478″	87°54′59.209″	352
MTU1	Houghton	MTU Trails	2, Arcadian-Michigamme sandy loams	*Quercus*, *Acer*, *Pinus*	Sugar Maple-Hemlock forest	47°6′24.649″	88°32′51.209″	266

Forest type describes the original forest type based on an interpretation of the 1816–1856 general land office surveys (Albert and Comer 2008)

[a] *Q. ellipsoidalis*. Soil type and drainage class according to the USDA Natural Resources Conservation Service's Web Soil Survey (http://websoilsurvey.nrcs.usda.gov/app/HomePage.htm)

1, excessively drained; 2, well drained; 3, moderately well drained

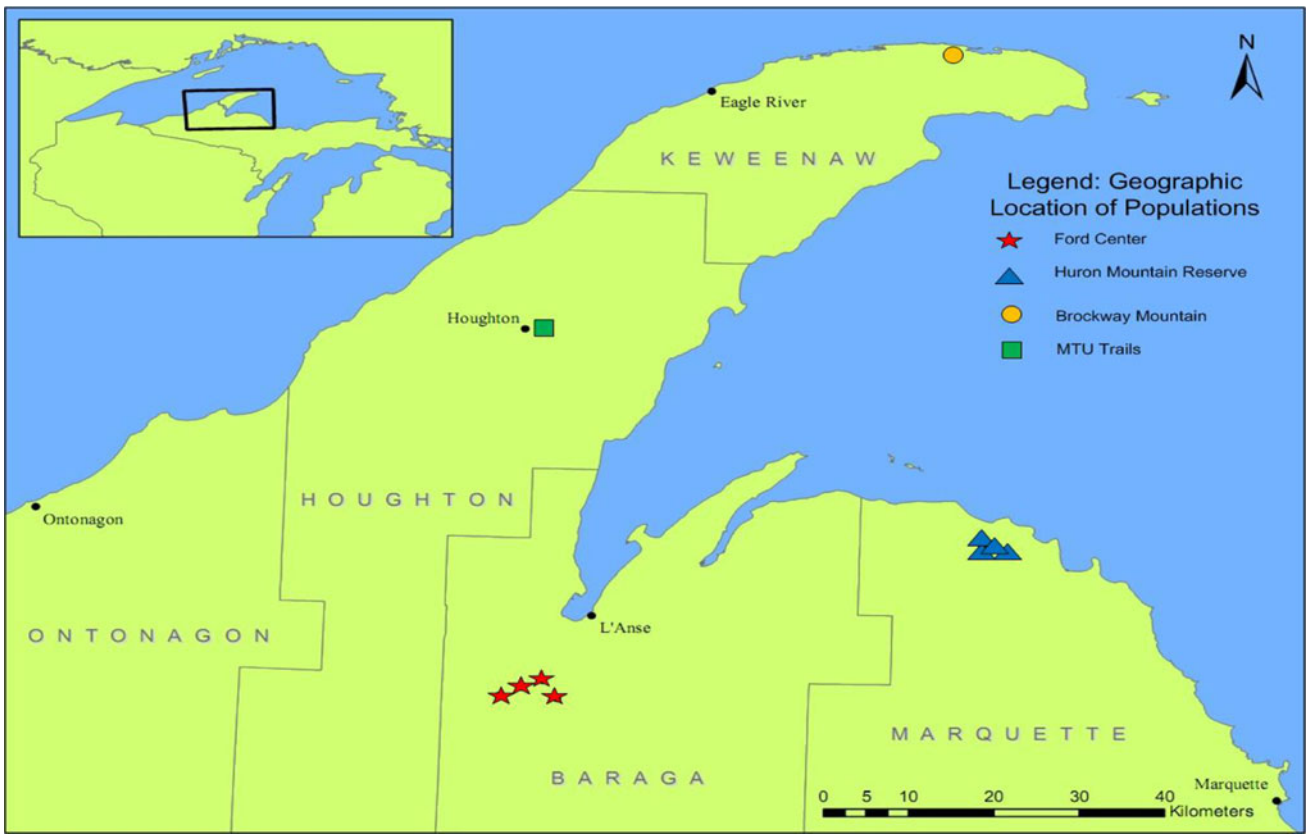

Fig. 1 Sample locations on the Upper Peninsula in Michigan. Site abbreviations as in Table 1

2 % per population. On average, 80 % of the individuals in *Q. ellipsoidalis* populations (FC-C, FC-E) that were included in the present study were identified as *Q. ellipsoidalis* and 17 % as *Q. ellipsoidalis* introgressive forms, while 92 % of trees were identified as *Q. rubra* and 7 % as *Q. rubra* introgressive forms in the *Q. rubra* populations (see also Lind and Gailing, unpublished data). Genetic assignment analyses were performed with the program Structure 2.2 (Pritchard et al. 2000), which uses Bayesian inference to evaluate the probability of each genotype to belong to *K* populations (species). "Pure" species, introgressive forms, and F_1 hybrids were defined as having a proportion of ancestry of ≥90 %, 0.61 to 0.89, and 0.4 to 0.6 in one species. More details of the genetic assignment analysis are given in Lind and Gailing (unpublished data).

Leaf morphological measurements

For each of the 434 trees, the four largest leaves were measured at 13 leaf characters (12 dimensional and 1 counted character) that were used to discriminate between *Q. rubra* and *Q. ellipsoidalis* by Jensen et al. (1993) (Table 2). Since most leaf dimensional characters showed strong correlation with leaf blade length (Table 3), four additional leaf shape characteristics (ratios of dimensional leaf characters) were calculated that describe shape differences between *Q. rubra* and *Q. ellipsoidalis* (Table 2; Fig. 2).

Genetic marker analyses

Fifteen molecular markers were amplified in all 434 trees to investigate genetic differentiation between populations and to assign individual samples to species (Lind and Gailing, unpublished data). These included seven expressed sequence tag–simple sequence repeat (EST–SSR) markers originally developed for *Q. robur* (Durand et al. 2010), seven simple sequence repeat (SSR) markers developed for *Q. rubra* (Aldrich et al. 2002; Sullivan et al., submitted), and the *Q. robur* microsatellite ZagQpZag15 (Steinkellner et al. 1997). DNA was extracted using the DNeasy96 Plant Kit (Qiagen) following the manufacturer's instructions and amplified using polymerase chain reaction (PCR) conditions as described previously (Aldrich et al. 2002; Durand et al. 2010; Lind and Gailing, unpublished data) on the Gene Amp PCR system 2700 (Applied Biosystems). Fragments were separated on the ABI 3730 capillary sequencer with the GS500LIZ size standard with resolution <0.5 bp and analyzed with GeneMapper 4.0 software (Applied Biosystems). As all repeats were at least

Table 2 Leaf morphological characters used in the morphological analysis

Leaf characteristic	Abbreviation	Landmarks used in calculation
Leaf blade length	LBL	1-11
Blade width defined by basal lobe pair	LBWB	2-12
Leaf blade width defined by middle lobe pair	LBWM	5-14
Leaf blade width defined by apical lobe pair	LBWA	8-16
Interval between basal pair of sinuses	INTB	4-13
Interval between middle pair of sinuses	INTM	7-15
Interval between apical pair of sinuses	INTA	10-17
Interval between center vein intersections	CENTER	6-9
Interval between apical vein intersections	APEX	9-11
Angle 1	ANG1	1-3-2
Angle 2	ANG2	11-6-5
Angle 3	ANG3	11-9-8
Number of bristle tips	NBT	NA
Ratio of INTB to INTM	INTB/INTM	NA
Ratio of LBWM to INTM	LBWM/INTM	NA
Ratio of LBL to INTM	LBL/INTM	NA
Ratio of LBL to APEX	LBL/APEX	NA

See Fig. 2 for location of landmarks. The characters were measured according to Jensen et al. (1993). Ratios were calculated based on these measurements

NA not applicable

two bases, unambiguous attribution to loci was possible. Pairwise genetic differentiation values (pairwise F_{ST} values) were calculated in GeneAlEx 6.2 (Peakall and Smouse 2006).

Data analysis

Hierarchical clustering, discriminant analysis (DA), and principal component analysis (PCA) were performed with the program WINSTAT (Fitch 2006). Analyses were performed on the directly measured leaf characters as used by Jensen et al. (1993) to discriminate between *Q. rubra* and *Q. ellipsoidalis*. Since most leaf dimensional characters showed strong correlation with leaf blade length (LBL), all analyses were also performed on all derived leaf characters including directly measured leaf characters and shape characters (Table 2). Ward's hierarchical clustering method was applied to identify clusters using standardized squared Euclidean distances. A DA with equal prior probabilities was performed to separate clusters and to calculate standardized coefficients of the discriminant function. A PCA was performed to find the independent factors underlying the leaf morphological variables using Varimax factor rotation. The analysis of communalities was continued until residues were smaller than 0.05.

Table 3 Correlation of leaf characters with lamina length (LBL)

	Coefficient of correlation
LBWB	0.569324
LBWM	0.78072
LBWA	0.689782
INTB	0.70236
INTM	0.79786
INTA	0.693321
CENTER	0.306096
APEX	0.700942
ANG1	0.592445
ANG2	0.916884
ANG3	0.758573
NBT	0.243627
INTB/INTM	−0.19071
LBWM/INTM	−0.36607
LBL/INTM	−0.29508
LBL/APEX	−0.29891

$p < 0.001$ for all characters

Differentiation among populations at leaf morphological characters was analyzed with a one-factor analysis of variance (ANOVA). The Student–Newman–Keuls test was used to test for critical differences in means between populations and to identify homogenous subsets. Interspecific differentiation was calculated between populations FC-C/FC-E (*Q. ellipsoidalis*) and FC-A/FC-B (*Q. rubra*) in one region to control for climatic effects on leaf characters. The differences of populations in leaf morphology were visualized in an unweighted pair group method with arithmetic means (UPGMA) dendrogram using NEIGHBOR in the program Phyllip 3.68 (Felsenstein 1989) based on differences in factors scores of factor 1 of the principal component analysis 1.

Leaf morphological distance matrices on the one hand were compared with geographic and genetic distances on

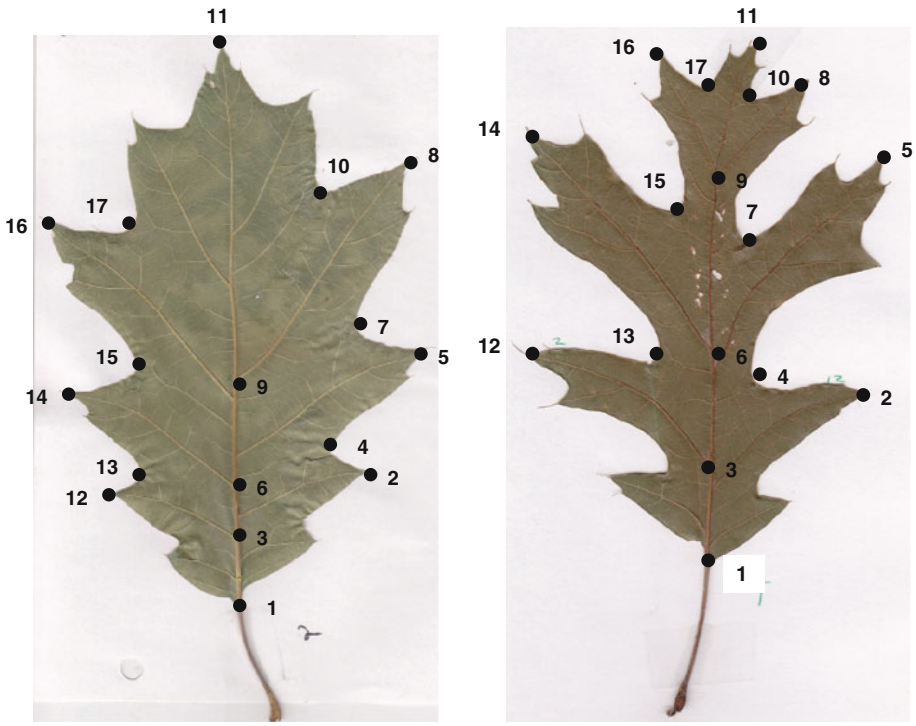

Fig. 2 Leaves of *Quercus rubra* (from stand FC-B) and *Q. ellipsoidalis* (stand FC-C) showing landmarks for leaf morphological measurements according to Jensen et al. (1993). Leaf dimensional and leaf shape characters are explained in Table 2

the other hand using a Mantel test as implemented in GenAlEx 6.2 with 999 permutations (Peakall and Smouse 2006).

Results

Correlation of leaf characters with leaf size

All dimensional leaf variables with the exception of CENTER showed strong correlation ($r > 0.5$) with leaf blade length (LBL). Number of bristle tips (NBT) and calculated leaf shape characters showed a lower, but still significant correlation with leaf size (Table 3).

Principal component analysis (PCA)

PCA using the 13 directly measured leaf variables (PCA1) as described by Jensen et al. (1993) to discriminate between *Q. rubra* and *Q. ellipsoidalis* revealed two factors explaining 77.1 % of the total variation (factor 1: 53.0 %; factor 2: 24.1 %). Factor 1 showed strong correlation ($r \geq 0.80$) with leaf dimensional characters LBWA, INTA, APEX, and ANG3 and with leaf shape characteristic LBL/APEX; factor 2 was strongly correlated with LBWB, LBWM, and ANG1 (Table 4).

A second PCA (PCA2) including leaf shape characteristics (ratios, see Table 2) revealed three factors separating factor 1 of PCA1 into two factors (factor 1 and 3). The three factors explained 81.7 % of the total variation (factor 1: 46.1 %; factor 2: 25.9 %; factor 3: 9.7 %) with factor 3 accounting mainly for leaf shape variation (leaf dissection, LBWM/INTM, LBL/INTM) (Fig. 2; Table 4).

Even though there was no clear-cut separation among all populations, *Q. ellipsoidalis* populations FC-C and FC-E grouped together and separate from the other populations in both analyses (PCA1, PCA2; see Supplementary Fig. 1).

Cluster and discriminant analyses

Cluster analysis based on the 13 directly measured leaf characters as described by Jensen et al. (1993) resulted in two major clusters (Fig. 3a). The DA revealed strong separation between groups (Mahalanobis distance between groups 2.693, $p = 5 \times 10^{-87}$), and the discriminant function explained 100 % of the grouping variables' variance (eigenvalue 1.806, $p = 4 \times 10^{-87}$). Based on the discriminant function, 95.12 % of the trees were classified correctly. ANG2 was the most important variable of the discriminant function (standardized coefficient of the discriminant function 0.455), showing strong separation between clusters (mean cluster 1: 14.52 cm; mean cluster 2: 19.40 cm; $t = -24.4$, $p = 8.6 \times 10^{-84}$). The absolute frequency of trees assigned to cluster 1 and 2 is shown in Fig. 4a for each population. All mean values for dimensional leaf characters were significantly lower for cluster 1, but showed significantly higher ratios for LBWM/INTM, LBL/INTM (more deeply dissected leaves), and

Table 4 Pearson's correlation coefficients between leaf morphological traits and factors of the two principal component analyses (PCA)

	PCA1[a]		PCA2		
	Factor 1	Factor 2	Factor 1	Factor 2	Factor 3
LBL	0.72	0.62	0.59	0.70	−0.23
LBWB	0.00	**0.96**	−0.13	**0.94**	−0.09
LBWM	0.41	**0.82**	0.33	**0.87**	−0.06
LBWA	**0.95**	−0.00	**0.91**	0.12	−0.22
INTB	0.35	0.79	0.06	0.75	−0.59
INTM	0.74	0.47	0.47	0.46	−0.71
INTA	**0.91**	0.04	0.75	0.10	−0.51
CENTER	−0.33	0.76	−0.35	0.74	0.21
APEX	**0.95**	0.01	**0.93**	0.15	−0.15
ANG1	−0.01	**0.94**	−0.18	**0.90**	−0.20
ANG2	0.70	0.63	0.64	0.73	−0.08
ANG3	**0.97**	0.09	**0.93**	0.22	−0.20
NBT	0.11	0.31	0.14	0.34	0.11
INTB/INTM	−0.63	0.46	−0.66	0.44	0.26
LBWM/INTM	−0.59	0.06	−0.31	0.13	**0.90**
LBL/INTM	−0.47	−0.14	−0.19	−0.04	**0.92**
LBL/APEX	**−0.80**	0.33	**−0.86**	0.23	0.12

[a] PCA1 was performed using only directly measured characters. PCA2 was performed using all characters, including ratios

Bold indicates $r \geq 0.80$

LBL/APEX typical for *Q. ellipsoidalis* leaves ($p < 0.0001$, independent t test; see also Fig. 2). All but one tree of *Q. ellipsoidalis* populations FC-C and FC-E growing on very dry and sandy soils belonged to cluster 1, while all other populations showed a mixture of trees belonging to cluster 1 and cluster 2. Of these, only population HMR-MI (shrubby trees on granite rocks) showed a majority of trees belonging to cluster 1, the remaining populations being mainly composed of cluster 2 trees (Fig. 4a).

The cluster analysis including leaf shape characteristics revealed three clusters separating trees from stands FC-C and FC-E clearly from the remaining populations (eigenvalue 2.13; percentage variance explained: 53.4 %; $p = 4 \times 10^{-86}$; Figs. 3b, 4b) with leaf blade length (LBL) (standardized coefficient of the discriminant function −0.917) and LBL/APEX (standardized coefficient of the discriminant function 1.007) showing the strongest discrimination between groups. Highly significant differences between clusters were found for leaf blade length (LBL) (cluster 1: 15.87 cm; cluster 2: 14.55 cm; cluster 3: 10.91 cm; $p < 0.0001$) and LBL/APEX (cluster 1: 2.18; cluster 2: 1.66; cluster 3: 2.55; $p < 0.0001$). The majority of individuals from FC-C and FC-E (*Q. ellipsoidalis* populations) grouped into leaf cluster 3, while very few of the trees from the other (*Q. rubra*) populations grouped into this cluster (Fig. 4b). According to the discriminant function, 91.4 % of the trees were classified correctly.

Good but incomplete concordance between genetic and leaf morphological assignment was found. Thus, 87.7 % of individuals genetically classified as *Q. ellipsoidalis* grouped into *Q. ellipsoidalis* leaf morphological cluster 3, 6.7 % into *Q. rubra* cluster 2, and 5.6 % into *Q. rubra* cluster 1. For individuals that were genetically classified as *Q. rubra*, 48.1 % grouped into leaf morphological cluster 1, 49.4 % into cluster 2, and 2.5 % into cluster 3. While most individuals were classified as "pure" species (80 % for *Q. ellipsoidalis* and 92 % for *Q. rubra* populations), a total of 18 *Q. ellipsoidalis* introgressive forms, 24 *Q. rubra* introgressive forms, and 4 F_1 hybrids were identified by the genetic assignment analysis (Lind and Gailing, unpublished data). Most of the *Q. ellipsoidalis* introgressive forms were assigned by leaf morphology as *Q. ellipsoidalis* (72.2 %), while the remaining 27.8 % grouped into *Q. rubra* cluster 2. Likewise, the vast majority of *Q. rubra* introgressive forms were identified by leaf morphology as *Q. rubra* (cluster 1: 58.3 %; cluster 2: 33.3 %) and only 8.4 % grouped into *Q. ellipsoidalis* cluster 3. Among the four F_1 hybrids two individuals grouped into cluster 1 and one individual grouped into cluster 2 and 3, respectively. These results suggest that leaf morphological variation was mainly caused by high within-species variation (hypothesis 2) and to a lesser degree by phenotypic differences between the two hybridizing species *Q. rubra* and *Q. ellipsoidalis* (hypothesis 1).

Differentiation between populations and species

Large variation in leaf morphological characters was found within and among populations. The one-factor ANOVA showed significant variation among populations for all

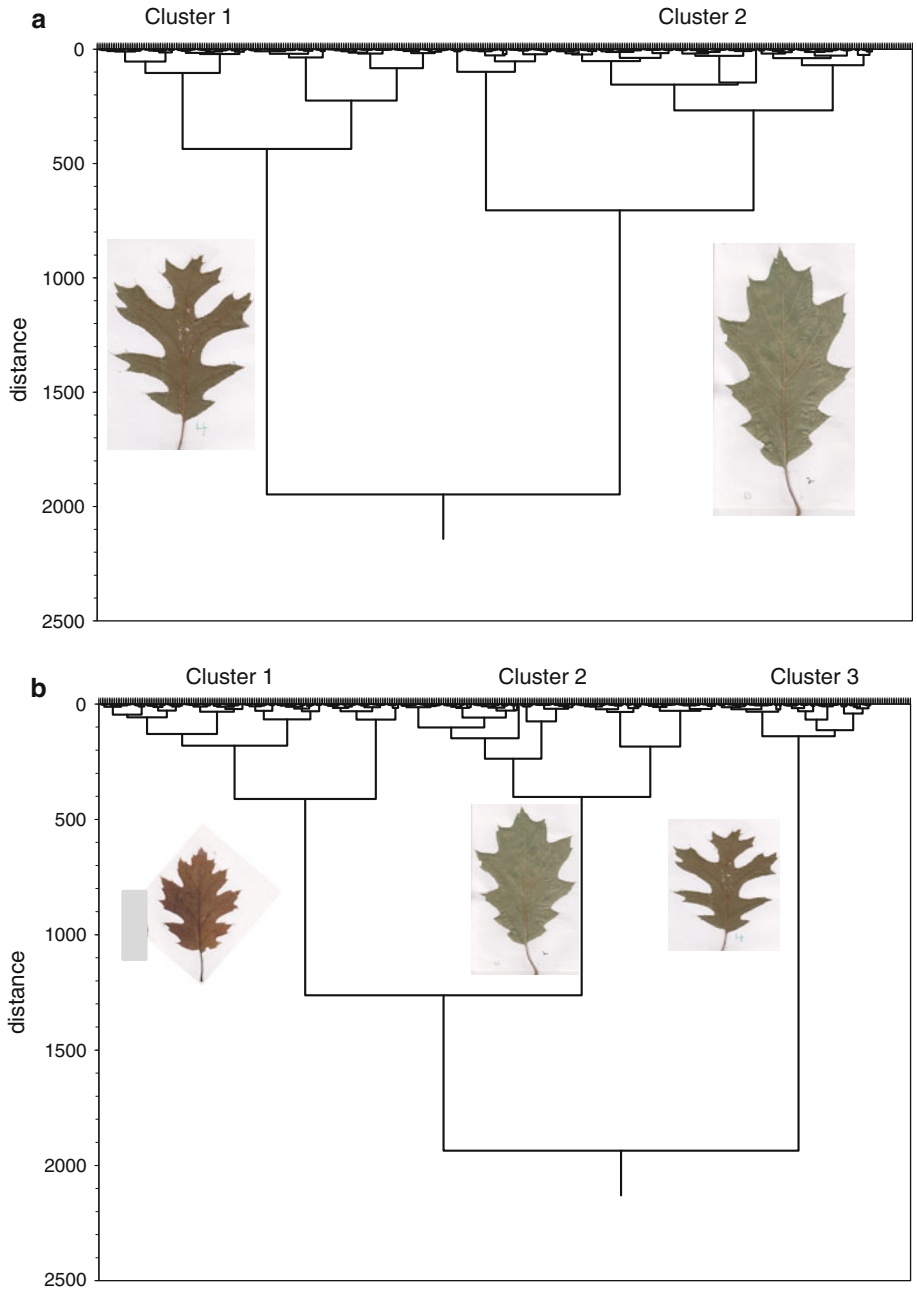

Fig. 3 Cluster analysis using Ward's method at 13 leaf dimensional characters (**a**) and at 17 leaf dimensional and leaf shape characters (**b**). Representative leaf samples for each cluster are shown

characters with an average differentiation of 42.3 % (Tables 5, 6). In Baraga County, where both species co-occur in close proximity (populations FC-A, FC-B, FC-C, and FC-E), the average differentiation between species was 39.2 % ($p < 0.0001$). For leaf characters INTM, LBL, and ANG3 and factor 1 of PCA1, more than 60 % of the variation was found between species in neighboring populations FC-C/FC-E (*Q. ellipsoidalis*) and FC-A/FC-B (*Q. rubra*) (Table 5). When only the eight *Q. rubra* populations were analyzed, the percentage of differentiation between populations was lower for nearly all characters within an average of 25.0 % of the variation being distributed among populations (Table 5). For example, the percentage of variation distributed among populations dropped from 47.5 to 14.2 % for leaf blade length (LBL), from 47.1 to 12.6 % for ANG2, from 50.4 to 22.5 % for APEX, and from 51.1 to 19.6 % for ANG3 when *Q. ellipsoidalis* populations were excluded from the analysis.

The population pair FC-C and FC-E (*Q. ellipsoidalis*) grouped together for 15 out of 17 characters and formed a single homogenous group significantly differentiated from the other populations at factor 1 (PCA1) and at eight leaf dimensional characters (LBL, LBWM, INTB, INTM, APEX, ANG2, and ANG3) (Table 6).

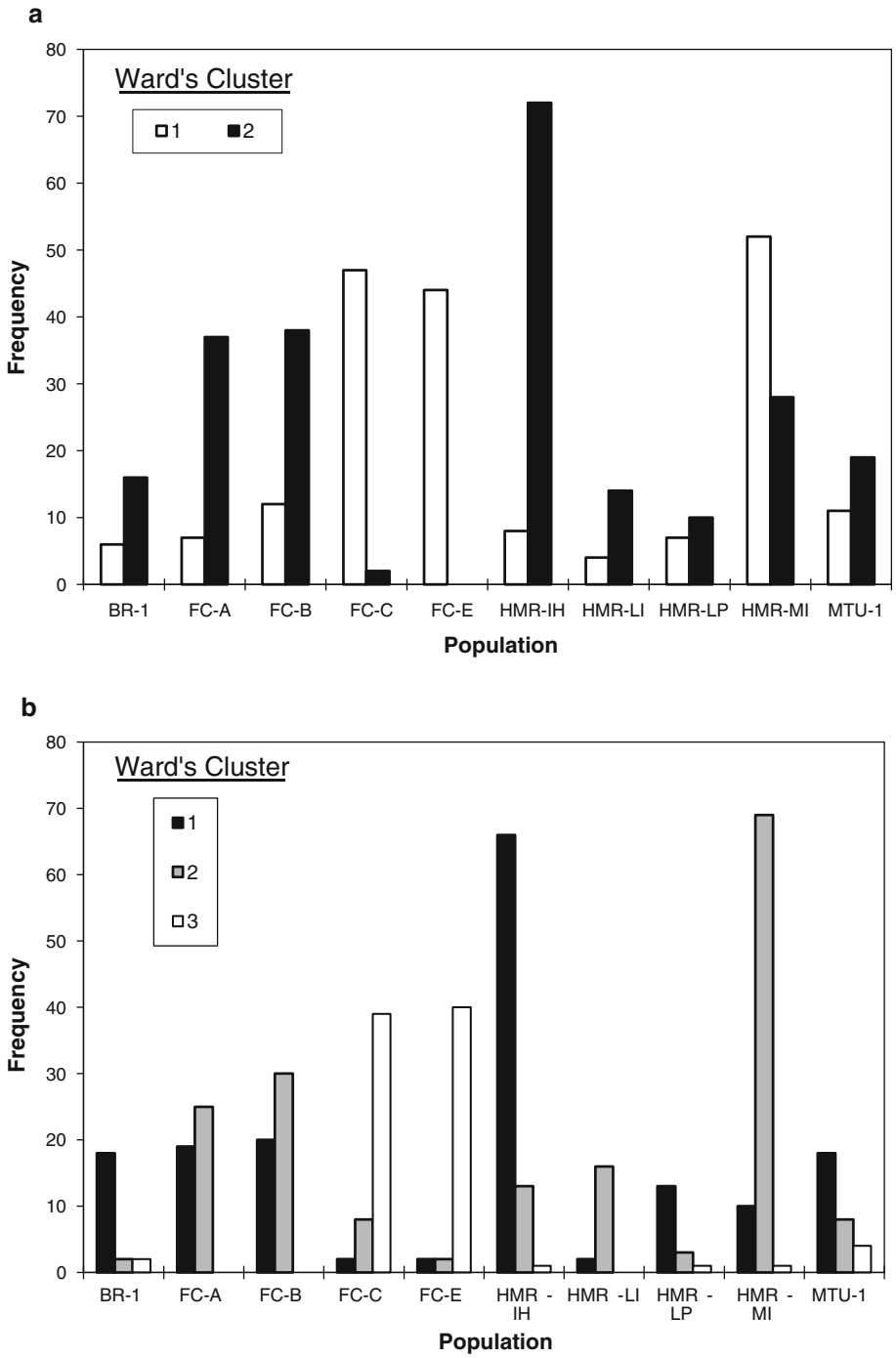

Fig. 4 Absolute frequencies of trees per population assigned to clusters based on 13 (**a**) and 17 (**b**) leaf morphological characters

A UPGMA tree based on differences in factor 1 of PCA1 between populations illustrates the similarity of populations FC-C and FC-E and their separation from the neighboring populations FC-A and FC-B (Fig. 5).

Correlation of phenotypic with geographic and genetic distances

None of the factors of the PCA showed significant correlation with the geographic distance of populations. Also genetic distance at the 15 microsatellite markers was not correlated with geographic distance. Strong and positive correlation was found between genetic distance at microsatellites (F_{ST}) and leaf morphological distance (factor 1 of PCA1) for all populations ($R^2 = 0.4833$, $p < 0.0001$) and after exclusion of *Q. ellipsoidalis* populations FC-C and FC-E ($R^2 = 0.419$, $p < 0.001$). Especially population pairs from different species BR/FC-E and MTU/FC-E showed a lower leaf morphological differentiation as expected by their genetic distance (Fig. 6). Also factor 1 of PCA2

Table 5 Analysis of variance of leaf morphological traits for *Q. rubra*/*Q. ellipsoidalis* populations

Trait	% Variation between populations	% Variation between species	% Variation between *Q. rubra* populations
LBL	47.5*	61.1*	14.2*
LBWB	51.9*	18.6*	47.8*
LBWM	42.2*	35.8*	24.9*
LBWA	49.7*	59.3*	23.2*
INTB	49.3*	49.7*	27.4*
INTM	50.4*	63.3*	22.9*
INTA	48.0*	56.2*	28.1*
CENTER	23.4*	2.0^{ns}	26.5*
APEX	50.4*	54.6*	22.5*
ANG1	48.7*	19.8*	44.8*
ANG2	47.1*	57.6*	12.6*
ANG3	51.1*	61.5*	19.6*
NBT	13.0*	6.2*	8.5*
INTB/INTM	25.2*	9.4*	31.0*
LBWM/INTM	33.6*	42.9*	27.4*
LBL/INTM	27.9*	33.0*	18.6*
LBL/APEX	47.5*	35.8*	38.8*
Factor 1 (PCA1)	54.0*	62.3*	53.5*

Factor 1 of principal component analysis 1 (directly measured characters) explains 53.0 % of the total phenotypic variance. Differentiation between species was calculated within one region for population pairs FC-C/FC-E (*Q. ellipsoidalis*) and FC-A/FC-B (*Q. rubra*)

df (among populations) = 9, df (within populations) = 424, df (between species) = 1, df (within species) = 185

ns not significant

* $p < 0.0001$

($R^2 = 0.22$, $p < 0.001$) and factor 3 of PCA2 ($R^2 = 0.19$, $p < 0.001$) showed significant correlations with genetic distance, while no such correlation was found for factor 2 (PCA1, PCA2). For leaf blade length (LBL) and LBL/APEX, which showed the strongest discrimination between clusters 1 and 2 (*Q. rubra* clusters) and cluster 3 (*Q. ellipsoidalis* cluster), differences were significantly correlated with genetic distances (LBL: $R^2 = 0.45$, $p < 0.001$; LBL/APEX: $R^2 = 0.23$, $p < 0.001$) for all populations, but not when *Q. ellipsoidalis* populations were excluded from the analysis ($R^2 = 0.014$, n.s.; LBL/APEX: $R^2 = 0.18$, n.s.).

Discussion

When only leaf characters according to Jensen et al. (1993) were used in the cluster analysis, all but one sample from *Q. ellipsoidalis* stands were assigned to one leaf cluster, however a large number of individuals from *Q. rubra* populations especially from population HMR-MI consisting of shrubby trees on granite rocks also grouped into this cluster. Adding four additional leaf shape characteristics allowed assigning most of the *Q. ellipsoidalis* samples to a distinct cluster, while most *Q. rubra* samples grouped into two other clusters. Thus, the inclusion of leaf shape characters allowed for a better separation between *Q. rubra* and *Q. ellipsoidalis*.

Even though *Q. ellipsoidalis* populations are differentiated from *Q. rubra* populations based on these 17 leaf morphological characters, the distinction is not clear-cut and different leaf types and variation in leaf morphologies were observed within populations and species (see also Supplementary Fig. 1). On the other hand, the genetic assignment analysis of individuals to species at microsatellite markers identified most of the individuals in the *Q. ellipsoidalis* populations as *Q. ellipsoidalis* in the present sample (80 % *Q. ellipsoidalis*, 17 % *Q. ellipsoidalis* introgressive forms, 2 % *Q. rubra* introgressive forms, 1 % hybrids) and most of the individuals in the *Q. rubra* populations as *Q. rubra* (92 % *Q. rubra*, 7 % *Q. rubra* introgressive forms, 0.6 % F_1 hybrids, 0.4 % *Q. ellipsoidalis* introgressive forms) (see also Lind and Gailing, unpublished data). A Mantel test of genetic distance at genetic markers against leaf morphological differences (factor 1 of PCA1) showed strong and highly significant correlation for all populations ($R^2 = 0.483$, $p < 0.001$) and after exclusion of the two *Q. ellipsoidalis* populations ($R^2 = 0.419$, $p < 0.001$), suggesting genetic and environmental effects on leaf character differences and considerable genetic variation for leaf morphological characters not only between species but also in *Q. rubra*. Thus, the high variation in leaf morphological characters within populations is mainly the product of high phenotypic variation within species in response to the microenvironment (phenotypic plasticity) and of genetic variation for leaf morphological characters within species (hypothesis 2), and not the product of phenotypic differences between two hybridizing species with incomplete reproductive isolation (hypothesis 1). Deviations from the linear relationship between genetic distance at microsatellite markers and leaf morphological differences suggest environmental effects on the expression of leaf morphological characters, but might also be indicative of stabilizing or directional selection on genes underlying these traits in different populations (see Fig. 6).

Leaf blade length (LBL) and LBL/APEX (see Table 2; Fig. 2) showed the highest discrimination between leaf clusters 1 and 2 ("*Q. rubra* clusters") and cluster 3 ("*Q. ellipsoidalis* cluster"). Differences in LBL and LBL/APEX between *Q. rubra* populations (14.2 and 38.8 % differentiation) seem to be mainly due to phenotypic plasticity, as shown by the nonsignificant correlation

Table 6 Population means for leaf morphological characters (standard deviations in parentheses)

Population	*n*	LBL	LBWB	LBWM	LBWA	INTB	INTM	INTA	CENT-ER	APEX	ANG1	ANG2	ANG3	NBT	INTB/INTM	LBWM/INTM	LBL/INTM	LBL/APEX
BR1[a]	22	15.27	10.26	11.56	7.42	4.67	4.10	2.68	4.04	6.96	7.59	18.64	12.33	23.72	1.15	2.91	3.84	2.23
		(1.78)	(1.45)	(1.70)	(2.61)	(0.83)	(0.85)	(0.52)	(0.79)	(1.24)	(0.91)	(2.50)	(2.15)	(4.82)	(0.12)	(0.64)	(0.74)	(0.32)
FC-A[b]	44	16.03	8.02	10.82	9.12	5.18	5.95	4.59	2.94	8.94	6.80	18.34	15.13	29.46	0.87	1.86	2.77	1.90
		(2.03)	(2.36)	(1.68)	(1.81)	(1.44)	(1.19)	(1.21)	(0.64)	(2.76)	(2.39)	(1.95)	(2.77)	(8.32)	(0.16)	(0.33)	(0.50)	(0.43)
FC-B[b]	50	15.55	8.12	10.56	8.69	4.82	5.34	4.14	2.99	8.50	6.58	18.26	15.00	29.44	0.91	2.02	2.98	1.89
		(2.14)	(2.21)	(1.83)	(2.08)	(1.09)	(1.11)	(1.24)	(0.64)	(1.97)	(2.01)	(2.58)	(3.15)	(7.18)	(0.16)	(0.35)	(0.43)	(0.33)
FC-C[c]	49	11.21	5.90	8.22	5.01	2.91	3.06	2.24	3.23	4.79	4.66	13.37	8.81	21.99	0.98	2.86	3.90	2.47
		(1.94)	(1.63)	(1.76)	(1.84)	(0.80)	(0.95)	(0.88)	(0.69)	(1.52)	(1.30)	(2.37)	(2.69)	(5.91)	(0.18)	(0.82)	(1.03)	(0.53)
FC-E[c]	44	10.86	6.68	8.32	4.33	2.97	2.91	1.89	3.05	4.35	5.27	13.10	8.09	29.82	1.03	2.93	3.83	2.54
		(1.32)	(1.00)	(1.14)	(0.92)	(0.64)	(0.55)	(0.39)	(0.47)	(0.77)	(0.88)	(1.63)	(1.49)	(5.58)	(0.17)	(0.53)	(0.68)	(0.31)
HMR-IH[d]	80	15.93	10.37	12.48	8.14	5.29	5.27	3.44	3.57	7.87	8.17	19.49	14.29	31.38	1.01	2.44	3.10	2.06
		(2.09)	(1.37)	(1.71)	(1.78)	(0.97)	(1.08)	(0.77)	(0.69)	(1.45)	(1.50)	(2.43)	(2.61)	(6.15)	(0.12)	(0.44)	(0.47)	(0.26)
HMR-LI[e]	18	15.83	6.04	10.03	11.09	4.13	5.20	4.88	2.60	10.68	4.41	18.99	17.83	25.50	0.81	1.98	3.10	1.51
		(2.25)	(1.50)	(1.28)	(2.03)	(0.67)	(0.99)	(1.24)	(0.74)	(2.21)	(1.29)	(2.58)	(3.32)	(8.19)	(0.13)	(0.40)	(0.46)	(0.21)
HMR-LP[a]	17	14.76	9.92	10.86	6.37	5.33	4.98	3.05	3.46	6.20	8.00	17.12	11.50	29.74	1.08	2.23	3.05	2.43
		(1.71)	(1.36)	(1.18)	(1.34)	(1.04)	(0.93)	(0.67)	(0.75)	(1.15)	(1.25)	(1.96)	(2.10)	(5.68)	(0.16)	(0.37)	(0.58)	(0.37)
HMR-MI[b]	80	13.93	6.29	9.89	9.32	3.80	4.37	3.60	2.70	8.86	4.47	17.16	15.22	25.12	0.88	2.32	3.26	1.62
		(1.83)	(1.42)	(1.71)	(2.12)	(0.64)	(0.82)	(0.82)	(0.68)	(1.88)	(1.15)	(2.41)	(3.18)	(13.14)	(0.13)	(0.50)	(0.56)	(0.27)
MTU1[ad]	30	14.58	8.93	10.60	6.81	5.09	4.87	3.14	3.32	6.84	7.40	17.21	12.39	27.19	1.06	2.21	3.04	2.17
		(2.48)	(1.71)	(1.70)	(1.85)	(0.92)	(1.01)	(0.82)	(0.81)	(1.55)	(1.47)	(2.88)	(2.73)	(5.64)	(0.16)	(0.29)	(0.38)	(0.31)
Total	434	14.30	7.94	10.37	7.70	4.35	4.59	3.36	3.15	7.46	6.23	17.15	13.19	27.60	0.96	2.39	3.28	2.04
		(2.69)	(2.35)	(2.14)	(2.62)	(1.29)	(1.36)	(1.23)	(0.77)	(2.46)	(2.09)	(3.20)	(3.87)	(8.65)	(0.16)	(0.61)	(0.71)	(0.47)

All values are in cm, apart from number of bristle tips (NBT) and ratios. The Student–Newman–Keuls test was used to test for critical differences in means between groups and to identify homogenous subsets. Data are shown for factor 1 of the principal component analysis 1 that explained 53.0 % of the total phenotypic variation. Letters a to e in column 1 characterize homogenous subsets. For 11 of the 13 directly measured leaf characters FC-C and FC-E grouped together, and for eight characters FC-C/FC-E were significantly separated from all other populations ($p < 0.01$). FC-C and FC-E grouped together for leaf shape characters LBWM/INTM, LBL/INTM, and LBL/APEX. FC-C, FC-E, and BRC formed distinct groups for LBWM/INTM and LBL/INTM

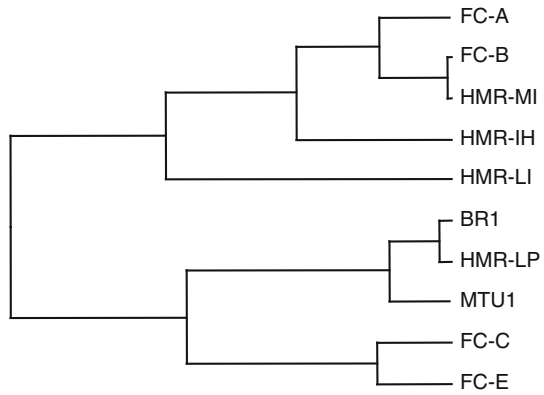

Fig. 5 UPGMA dendrogram based on a suite of related leaf morphological differences (factor 1 of PCA1 explaining 53 % of the total phenotypic variation) between populations

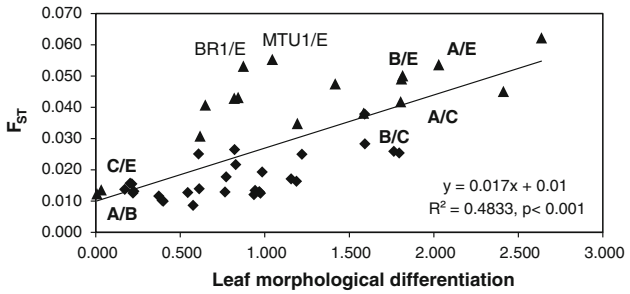

Fig. 6 Correlation between genetic distances between populations at 15 nuclear microsatellite markers (pairwise F_{ST} values) and leaf morphological differences (factor 1 of PCA1) using a Mantel test. *Triangles* show pairwise comparisons between stands on dry sandy soils (FC-C and FC-E) and other stands. Pairwise comparisons between adjacent stands FC-A (*A*), FC-B (*B*), FC-C (*C*), and FC-E (*E*) in the Baraga Plains region are highlighted in *bold*

between genetic differences (F_{ST}) and leaf differences between populations ($R^2 = 0.014$ for LBL, $R^2 = 0.18$ for LBL/APEX). However, genetic differentiation at a few genes with large phenotypic effects in combination with gene flow across the rest of the genome could also explain these results. On the other hand, differences in LBL and LBL/APEX between species *Q. rubra* and *Q. ellipsoidalis* reflect genetic differences and phenotypic plasticity between species, as shown by the positive correlation between genetic and leaf differentiation ($R^2 = 0.45$ for LBL, $R^2 = 0.23$ for LBL/APEX, $p < 0.001$). Common garden experiments in homogeneous environments are necessary to assess the relative importance of environmental and genetic effects in determining leaf morphological differences between species and among populations within species.

Deeply dissected and small leaves as found in *Q. ellipsoidalis* trees and the shrubby growth form on dry outwash sands might be an adaptation to lower water availability and higher soil evaporation in these open *Q. ellipsoidalis* stands on dry outwash sands. The following observations are consistent with a pattern of "isolation by adaptation" between species and among populations within species as the result of divergent selection (Nosil et al. 2009) on leaf characters or on adaptive characters associated with leaf morphology: (1) strong and positive association of genetic distance at microsatellite markers with leaf morphological differences, but absence of isolation by distance between populations and species at leaf morphological and at genetic markers, (2) relatively high differentiation between hybridizing species in contrasting environments at leaf morphological and genetic characters at a small geographic scale, and (3) much higher differentiation at quantitative leaf characters than at putatively neutral genetic markers.

The relatively low number of F_1 hybrids ($\leq$2.0 %) or introgressive forms ($\leq$17.0 % for *Q. ellipsoidalis*, $\leq$8 % for *Q. rubra*) in neighboring populations of *Q. rubra* (FC-A, FC-B) and *Q. ellipsoidalis* (FC-C, FC-E) that grow within the range of gene flow but on different soil types (xeric/mesic) (Lind and Gailing, unpublished data), and the strong differentiation of these adjacent populations at leaf morphological characters and other characters such as growth form and seed production, suggest the existence of such pre- and/or postzygotic barriers between *Q. rubra* and *Q. ellipsoidalis* populations. Also the increased genetic differentiation between *Q. ellipsoidalis* and *Q. rubra* stands in contrasting environments as observed at both nSSRs and EST–SSRs (Lind and Gailing, unpublished data) is expected even for neutral genetic markers as adaptive divergence increases, since effective gene flow is expected to decrease as the result of pre- or postzygotic isolation (Nosil et al. 2009). To distinguish between these two types of barriers, we have efforts underway examining the timing of vegetative bud burst (prezygotic barrier) and screening of survival and growth rates in seedlings (postzygotic barrier) in these species. Thus, in a common garden experiment under controlled non-drought stress conditions, *Q. ellipsoidalis* seedlings showed higher mortality and later bud burst than *Q. rubra* seedlings from a neighboring population (Gailing, submitted), suggesting prezygotic isolation and adaptive differences between species in the early seedling stage in which the highest viability selection is observed in forest trees (Jump et al. 2006; Müller-Starck 1985). Reciprocal transplants of *Q. rubra* and *Q. ellipsoidalis* seedlings between parental stands under natural conditions will be necessary to assess the effect of environment on fitness-related traits. A higher number of hybrids in the seedling generation than in the adult tree generation would indicate selection against hybrids (postzygotic barriers).

Pairs of neighboring populations of the same species or of hybridizing species within the range of gene flow in

contrasting environments (xeric/mesic), such as those identified in the current study, can serve as an experimental model system to search for genes with higher (signature of directional selection) or lower differentiation (signature of balancing selection) between populations/species than expected under selective neutrality (outlier loci). These outlier loci, especially when found across different populations pairs, have a potential role in local adaptation and/or prezygotic isolation (e.g., flowering time genes) (Nosil et al. 2009; Scotti-Saintagne et al. 2004).

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